



بسم الله الرحمن الرحيم



Sudan University of Science and Technology

College of Graduate Studies

**Isolation, Characterization and Biological
Activity of Flavonoids from Cordia Sinensis
and Acacia Orefota**

الفصل والتوصيف والفعالية البيولوجية للفلافونيدات لنباتي الأندراب واللحوت

**Thesis Submitted in Fulfillment of the Requirements
of the Ph.D. Degree in Chemistry**

By

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استعمال

قَالَ تَعَالَى:

﴿ أَلَمْ تَرَ أَنَّ اللَّهَ أَنْزَلَ مِنَ السَّمَاءِ مَاءً فَسَلَكَهُ يَنْبِيعَ فِي الْأَرْضِ
ثُمَّ يُخْرِجُ بِهِ زَرْعًا مُخْتَلِفًا أَلْوَانُهُ ثُمَّ يَهِيَجُ فَتَرَهُ مُصْفَرًّا ثُمَّ
يَجْعَلُهُ حُطَامًا إِنَّ فِي ذَلِكَ لَذِكْرًا لِأُولِي الْأَلْبَابِ ﴾

(الزمر: 21)

Dedication

To :

My parents

My wife and children

brothers and sisters .

Acknowledgement

I am grateful to AlmightyAllah for the will and blessings in completing this work.

I would like to express my thanks to Professor Mohamed Abdel Karim Mohamed, my supervisor, who gave me the chance to do this wonderful research. He also helped me in being engaged with up-to-date literature in the field of research. I also would like to thank my family and friends who helped me a lot in finalizing this research within the limited timeframe.

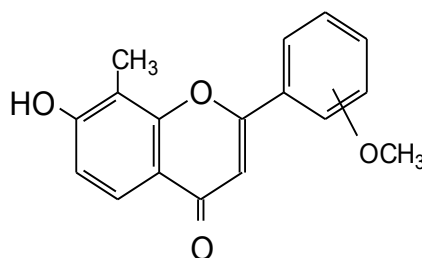
My special thanks are extended to the staff of chemistry department, Sudan University of Science and Technology, for their support and encouragement throughout my study. Also thanks to Engineer Balla Elbakry Daw El-Beit for his infinite support.

Abstract

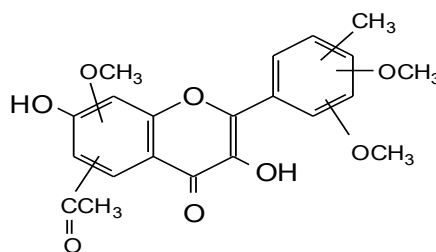
This study was designed to investigate the major flavonoids of two medicinal plants: *Cordia senensis* and *Acacia oerfota* which are widely used in Sudanese traditional medicine.

The bark of *Cordia senensis* was extracted with aqueous ethanol and the crude product was screened for secondary metabolites. Results showed the presence of flavonoids, tannins, alkaloids and sterols. Test for saponins was negative.

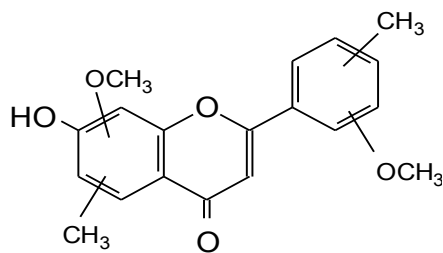
The crude extracts of *Cordia senensis* and *Acacia oerfota* were fractionated by different chromatographic techniques. After the usual workup compound I was isolated from *Cordia senensis*, while compounds II and III were isolated from *Acacia oerfota*.



Compound I



II



Compound III

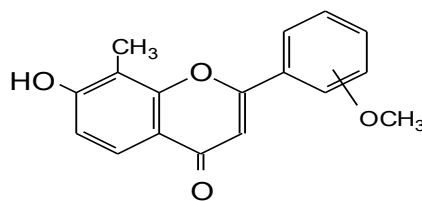
In cup plate diffusion bioassay, different concentrations of total extract of *Acacia oerofota* exhibited different antimicrobial responses against Gram positive and Gram negative bacteria, with zones of inhibition ranging from 6 to 13mm.

In addition, the extract showed good activity against *B. subtilis*, *P. aeruginosa* and *E. coli* with zones of inhibition of 13, 13 and 12 mm, respectively with minimum inhibitory concentrations (MIC) 62.5, 125 and 125 μg , respectively. However it showed moderate inhibitory activity against the Gram positive bacterial pathogen *S. aureus* with zone of inhibition 9 mm.

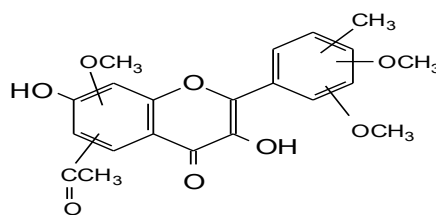
المستخلص

صمم هذا البحث لدراسة الفلافونيدات الرئيسية في نباتي : اللعوت والاندراب وهما من النباتات الهامة في الطب الشعبي بالسودان. اتضح من المسح الفيتوكيميائي للحاء نبات الاندراب وجود الفلافونيدات ، التتينات ، القلويدات والاسترولولات الا ان الصابونينات لم تكن موجودة.

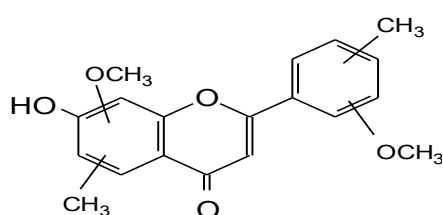
نقي مستخلصي النباتين بكروماتوغرافيا العمود ثم كروماتوغرافيا الطبقة الرقيقة حيث تم فصل المركب I من نبات الأندراب بينما تم فصل مركبي II و III من نبات اللعوت:



Compound I



II



Compound III

أختبر المستخلص الإيثانولي لنبات اللعوت حيث أظهر فعالية جيدة ضد عدة أنواع من البكتيريا.

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1. Introduction

1.1 General overview

Natural products are organic and inorganic compounds that are found in various natural sources plants, microbes and animals. Natural products can be entire organism (e.g. a plant, an animal or micro-organism), a part of an organism (e.g. leaves or flowers of a plant, an isolates animal organ) are extract of an organism or an exudates, or pure compound (e.g. alkaloids, coumarone, flavonoids, lignin's steroids and terpenoids) isolated from plants, animals or microorganisms. However, in practice, the term natural product refers to secondary metabolites, small molecules (molecular weight <1500 amu), produced by an organism, but not strictly necessary for the survival of the organism¹.

It has been estimated that well over 300, 00 secondary metabolites exist, and its thought that the primary function is to increase the likelihood of an organism survival by repelling or attracting other organisms. Alkaloids, such as morphine; eicosanoids, such as prostaglandin E; and antibiotics, such as erythromycin and the penicillin's, are good examples of secondary metabolites¹.

1.2 Classification of natural products

There is no rigid scheme for classifying natural products - their immense diversity in structure; function and biosynthesis are too great to allow them to fit neatly into a few simple categories. Initially, the natural products are classified into tow broad divisions: (i) primary metabolites (occur in all organism and (ii) secondary metabolites (occur only in certain organisms). The primary metabolites includes carbohydrates, amino acids, peptides, proteins, nucleic acids, lipids, and secondary metabolites includes classes of natural products: terpenoids and steroids, fatty acid-derived substances and polypeptide, alkaloids, nonribosomal polypeptides, and enzyme cofactors¹.

1.2.1 Terpenoids and Steroids

This includes a vast group of substances - more than 35,000 are known-derived biosynthetically from isopentenyl diphosphate. Terpenoids have an immense variety of apparently unrelated structures, while steroids are modified terpenoids, having a common tetracyclic carbon skeleton. These are biosynthesized from the triterpene, lanosterol¹.

1.2.2 Alkaloids

Like terpenoids, there is a large and diverse class of compounds, with more than 12,000 examples known at present. They contain a basic amine group in their structure and are derived biosynthetically from amino acids. (Precise Chemistry of Natural products and Heterocyclic Compounds)

1.2.3 Fatty acid-derived substances and polypeptides

About more than 10,000 are known, are biosynthesized from simple acyl precursors such as acetyl CoA, and methylmalonyl CoA. Natural products derived from fatty acids, such as the eicosanoid prostaglandin generally have most of the oxygen atoms removed, but polypeptides, such as the antibiotic erythromycin A, often have many oxygen substituents remaining¹.

Non-ribosomal polypeptides are peptide like compounds that are biosynthesized from amino acids by a multifunctional enzyme complex without direct RNA transcription. The penicillins are good example, but their chemistry is a bit complicated. Enzyme cofactors don't fit one of other general categories of natural products and are usually classed separately¹.

1.3 Natural products in medicine

Natural products have been a potential source of therapeutic agents for years. An impressive number of modern drugs have been derived from natural sources. Over the last century, a number of top selling drugs have

been developed from natural products. Anticancer drug-vincristine from *Vince rosea* and Taxol from *taxi* from *taxes brevifolia*, narcotic analgesic-morphine from *papaver somniferum*, antimalarial drug-artemisinin from *Artemisia annua*, and antibiotic penicillins from *penicillium* species are just a few examples. Apart from natural-product-derived modern-medicine, natural products are used directly in the natural pharmaceutical industry that is growing rapidly throughout the world¹.

1.4 Isolation of natural products

Secondary metabolites, with some exceptions, occur very less in amounts, about 0.01% of the dry weight of the plant. Extraction of 1 kg of dry plant material is likely to yield less than 100 mg of a natural product. These compounds may be unstable and present as part of a complex mixture. The isolation, separation and purification of these natural products require considerable skill. The source of a secondary metabolite requires proper identification and so a voucher specimen needs to be retained. Within the same species there are sometimes chemotypes, each with a particular composition. Some compounds are found in the roots, some are components of the bark, and other may be found insect antifeedants. Thus, the part of the plant and the place and date on which the plant was collected should all be recorded. Microorganisms are usually deposited in national culture collections. The production of microbial metabolites often depends upon the medium on which the microorganism is grown and on fermentation details. Some fungal metabolites are retained in the fungal mycelium, whilst others are excreted into the broth¹.

Insects, marine organisms and fungi that were collected in the wild may have stored and modified compounds which they had obtained from their food. Natural products may be obtained from the crushed biological material by extraction with a solvent such as petroleum ether, chloroform, ethyl acetate or methanol. Several solvents of increasing polarity may be

used. Thus lipid material (waxes, fatty acids, sterols, carotenoids and simple terpenoids) can be extracted with non-polar solvents such as petroleum ether, but more polar substances such as the alkaloids and glycosides are extracted with methanol, aqueous methanol or even hot water. Many alkaloids are present as their salts with naturally occurring acids such as tartaric acid¹.

Commercial extractions may use large quantities of plant material, and a range of different extraction procedures including steam distillation have been used. Recently, commercial procedures have been developed using supercritical carbon dioxide as a mild solvent, but because of the pressures involved this requires quite a complicated apparatus. The initial extraction is then followed by a separation into acidic, basic and neutral fractions. A typical solution of the extract in an organic solvent (such as ethyl acetate) is shaken with an inorganic base (such as aqueous sodium hydrogen carbonate) to remove the carboxylic acids as their water soluble sodium salts. The more weakly acidic phenols may only be extracted with a sodium hydroxide solution. Extraction of the original solution with an acid such as dilute hydrochloric acid removes the bases such as the alkaloids as their salts. The neutral compounds remain behind in the organic phase. The acids, the phenols may be recovered from the aqueous solution of their sodium salts by treatment with dilute hydrochloric acid and re-extraction with an organic solvent and the bases may be recovered by treatment of their salts with ammonia and re-extraction¹.

Although, some abundant natural products may be obtained merely by extraction, a simple fractionation or partition and crystallization, the majority are obtained after further careful chromatography. A typical example might involve chromatography on silica or alumina and with increasing concentrations of ethyl acetate in petroleum ether. The chromatographic separation may be monitored by a bioassay or by thin

layer chromatography (TLC). A number of useful reagents have been developed which produce colored TLC spots indicative of particular classes of compound.

In the course of a separation, reactions such as ester hydrolysis, autoxidation and rearrangement may occur, leading to the formation of artefacts. Hence efforts are made to ensure that a separation is chemically mild¹.

Natural products often co-occur in closely related series, for example as the mon-, di- and trihydroxy derivatives of the same parent compound. Since this relationship may facilitate structure determination, it is helpful to characterize and examine not only major product obtained from and isolation procedure but also the minor components.

Flavonoids are polyphenolic that are ubiquitous in nature. These are categorized according to chemical structure into flavonols, flavones, isoflavones, anthocyanidins and chalcones. Over 4,000 flavonoids have been identified, most of them occur in fruits. Vegetables and beverages (tea, coffee, beer, wine and fruit drinks) the flavonoids have aroused considerable interest recently because their potential beneficial effects on human health. They have been reported to have antiviral, Antiallergic, antiplatelet, anti-inflammatory, Antitumor and antioxidant activities. These are responsible for many of the plant colors that dazzle us with their brilliant shades of yellow, Orange and red¹.

Classified as plant pigments, were discovered in 1938 when a Hungarian scientist, Albert Szent-Gyorgyi used the term vitamin P to describe them. The chemistry of flavonoids is complicated, and within the non-technical term, flavonoids can be found with many different chemical groups of substances. As indicated above, and anthocyanidins. For example well known flavonols include quercetin, myricetin, and hesperidins, while well-known flavones include apigenin and, luteolin¹.

The study of flavonoid chemistry has emerged, like that of most natural products, from the search for new compounds with useful physiological properties. Semi synthetic endeavors of oligoflavonoids are in most instances confined to those substitution patterns exhibited by monomeric natural products that are available in quantities sufficient for preparative purposes. In order to alleviate these restrictions, several programs focusing on synthesis of enantiomeric pure flavonoid monomers have been undertaken. However, synthesis of the desired enantiomer in optically pure forms remains a daunting objective and is limited to only a few types of compounds. Chalcone epoxides, α - and β hydroxydihydrochalcones, dihydro-flavonols, flavan-3-ols, flavan-3,4-diols, isoflavans, isoflavanones, and pterocarpan thus far have been synthesized in reasonable yields and purity¹.

Flavonoids consist of a large group of polyphenolic compounds having a benzo- γ -pyrone structure and are ubiquitously present in plants. They are synthesized by phenylpropanoid pathway. Available reports tend to show that secondary metabolites of phenolic nature including flavonoids are responsible for the variety of pharmacological activities². Flavonoids are hydroxylated phenolic substances and are known to be synthesized by plants in response to microbial infection³. Their activities are structure dependent. The chemical nature of flavonoids depends on their structural class, degree of hydroxylation, other substitutions and conjugations, and degree of polymerization⁴. Recent interest in these substances has been stimulated by the potential health benefits arising from the antioxidant activities of these polyphenolic compounds. Functional hydroxyl groups in flavonoids mediate their antioxidant effects by scavenging free radicals and/or by chelating metal ions^{5,6}. The chelation of metals could be crucial in the prevention of radical generation which damage target biomolecules^{7,8}. As a dietary component, flavonoids are thought to have

health-promoting properties due to their high antioxidant capacity both in vivo and in vitro systems^{9,10}. Flavonoids have ability to induce human protective enzyme systems. The number of studies has suggested protective effects of flavonoids against many infectious (bacterial and viral diseases) and degenerative diseases such as cardiovascular diseases, cancers, and other age-related diseases^{2,9,10}. The mechanisms involved in protection provided by flavonoids are described separately in this review. Flavonoids also act as a secondary antioxidant defense system in plant tissues exposed to different abiotic and biotic stresses. Flavonoids are located in the nucleus of mesophyll cells and within centers of ROS generation. They also regulate growth factors in plants such as auxin¹¹. Biosynthetic genes have been assembled in several bacteria and fungi for enhanced production of flavonoids¹². This review deals with the structural aspects of flavonoids and their protective roles against many human diseases. Functions of flavonoids in plants and their microbial production have also been described.

1.5 Properties of flavonoids

- 1- These are crystalline substances with certain melting point.
- 2- These are soluble in water, dilute mineral acid, alkali, alcohol and ether.
- 3-Because of the two free electrons at the I-oxygen of pyrone ring, Flavonoids exhibit weak alkalinity and can form oxonium salt with strong acids like HCl and H₂SO₄, However, the obtained salt is extremely unstable and hydrolyzes immediately with the addition of water.
- 4-Flavones, flavonols and their glycosides are generally grayish yellow to yellow. Chalcones are yellow to orange yellow, flavanones and flavanonols have no conjugation system and thus are generally colorless.
- 5- These absorb wavelength near UV-region and this color is perceived by insects for pollination.

6- Flavones show 2 UV absorption bands: (a) 330-390 nm; (b) 250-270 nm. Therefore, can be distinguished from anthocyanins. Similarly, flavanols have 2 absorption bands: (a) 350-390 nm (due to absorption of cinnamoyl system in ring B); (b) 250-270 nm (due to absorption of benzoyl system in ring A)¹.

1.6 Flavonoid- rich foods and medicinal plants

Flavonoids are the most common and widely distributed group of plant phenolic compounds, occurring virtually in all plant parts, particularly the photosynthesizing plant cells. They are a major coloring component of flowering plants. Flavonoids are an integral part of human and animal diet. Some food sources containing different classes of flavonoids are given in Table 2. Being phytochemical, flavonoids cannot be synthesized by humans and animals¹³. Thus flavonoids found in animals are of plant origin rather than being biosynthesized in situ. Flavonols are the most abundant flavonoids in foods. Flavonoids in food are generally responsible for colour, taste, prevention of fat oxidation, and protection of vitamins and enzymes¹⁴. Flavonoids found in the highest amounts in the human diet include the soy isoflavones, flavonols, and the flavones. Although most fruits and some legumes contain catechins, the levels vary from 4.5 to 610 mg/kg¹⁵. Preparation and processing of food may decrease flavonoid levels depending on the methods used. For example, in a recent study, orange juices were found to contain 81–200 mg/L soluble flavanones, while the content in the cloud was 206–644 mg/L which suggest that the flavanones are concentrated in the cloud during processing and storage¹⁶. Accurate estimation of the average dietary intake of flavonoids is difficult, because of the wide varieties of available flavonoids and the extensive distribution in various plants and also the diverse consumption in humans¹⁷.

Table1: Food sources of some dietary flavonoids

Class	Flavonoid	Dietary Source	Reference
Flavanol	(+)- Catechin (-)- Epicatechin Epigallocatechin Chysin, Apigenin	Tea	[18]
Flavone	Rutin,Luteolin,and Luteolin glycoside.	Fruit skins, red wine, buckwheat, red pepper, and tomato skin.	[19-20]
Flavanol	Kaempferol,Quercetin, Myricetin,and Tamarixetin.	Onion, red wine, Olive, berries, and grapefruit.	[21]
Flavanone	Naringin, Naringnin, Taxifolin,and Hesperidin	Citrusfruit, grapefruits, and Oranges	[22-23]
Isoflavone	Genistin, Diadzin.	Soya bean	[24]
Anthocyanidin	Apigenidin, Cyanidin	Cherry, easberry, and Strawberry	[20-21]

Recently there has been an upsurge of interest in the therapeutic potential of medicinal plants which might be due to their phenolic compounds, specifically to flavonoids^{25,26}. Flavonoids have been consumed by humans since the advent of human life on earth, that is, for about 4 million years. They have extensive biological properties that promote human health and help reduce the risk of diseases. Oxidative modification of LDL cholesterol is thought to play a key role during atherosclerosis. The isoflavan glabridin, a major polyphenolic compound found in *Glycyrrhiza glabra* (Fabaceae), inhibits LDL oxidation via a mechanism involving scavenging of free radicals²⁷. Several

epidemiologic studies have suggested that drinking either green or black tea may lower blood cholesterol concentrations and blood pressure, thereby providing some protection against cardiovascular disease. Flavonoids are also known to influence the quality and stability of foods by acting as flavorants, colorants, and antioxidants^{28,29}. Flavonoids contained in berries may have a positive effect against Parkinson's disease and may help to improve memory in elderly people. Antihypertensive effect has been observed in total flavonoid fraction of *Astragalus complanatus* in hypertensive rats³⁰. Intake of antioxidant flavonoids has been inversely related to the risk of incidence of dementia³¹. Table(2) summarizes some of the medicinal plants rich in Flavonoid contents.

Table (2): Medicinal plants rich in flavonoid content

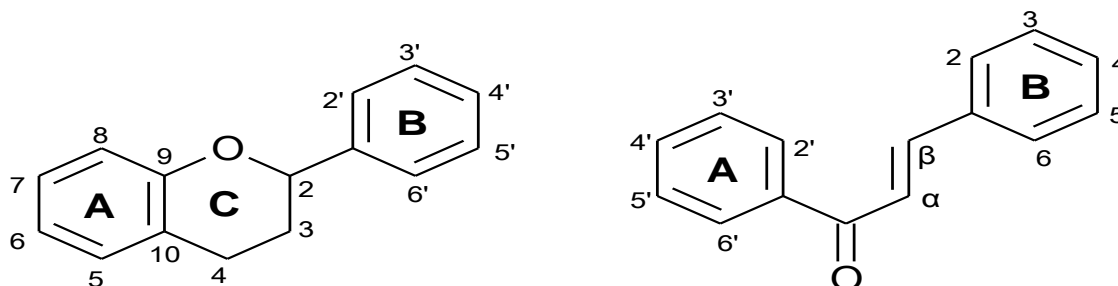
Plant	Family	Flavonoid	Reference
Aleovera	Asphodelaceae	Luteolin	32
Acalypha indica	Euphorbiaceae	Kaempferolglycoside	32
Azadirachta indica	Meliaceae	Quercetin	33
Andrographis paniculata	Acanthaceae	5-hydroxy-7,8-dimethylflavone	34
Bacopa moneirra	Scrophulariaceae	Luteolin	32
Betula monospermea	Butalaceae	Quercetin	34
Bauhinia monandra	Fabaceae	Genistein	35
Brysonima crassa	Fabaceae	Quercetin-3-O-rutinoside	35
Calendula officinalis	Malphigaceae	(+)- Catechin	36
Cannabis sativa	Compositae	Isoramnetin	34

Citrus medica	Rutaceae	Quercetin	34
Clerodendrum phlomidis	Verbenaceae	Hesperidin	32
Clitoria ternatea	Fabaceae	Pectolarigenin, Kaem-3- neohesperidoside	33
Glycyrrhiza glabra	Leguminosae	Liquiritin	37
Mimosa pudica	Mimosoideae	Isoquercetin	38
Limnophila indica	Scrophulariaceae	3,4- methylenedioxyflavone	38
Mentha logifolia	Lamiaceae	Luteolin-7-O-glycoside	39
Momordica charantia	Curcubitaceae	Luteolin	40
Oroxylum indicum	Bignoniaceae	Chrysin	38
Passiflora incarnate	Passifloraceae	Vitexin	34
Pongamia pinnata	Fabaceae	Pongaflavonol	41
Tephrosia purpurea	Fabaceae	Purpurin	38
Tilia cordata	Tiliaceae	Hyperoside	34

Solubility may play major role in the therapeutic efficacy of flavonoids. Low solubility of flavonoid aglycones in water coupled with its short residence time in the intestine as well as its lower absorption does not allow humans to suffer acute toxic effects from the consumption of flavonoids, with the exception of a rare occurrence of allergy. The low solubility of the flavonoids in water often presents a problem for its medicinal applications. Hence, the development of semi synthetic, water-soluble flavonoids, for example, hydroxyethylrutosides and inositol-2-phosphatequercetin, has been implicated for the treatment of hypertension and microbleeding⁴².

1.7 Classification of flavonoids

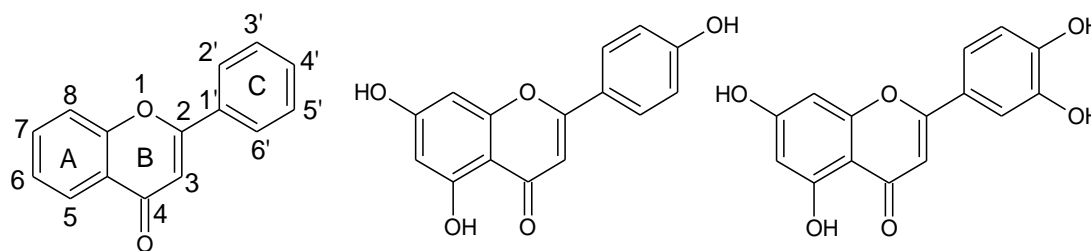
Chemically, the flavonoids are poly phenolic compounds possessing 15 carbon atoms; two benzene rings joined by a linear three carbon chain, as shown below:



The skeleton above can be represented as the C₆-C₃-C₆ system. The chemical structure of flavonoids are based on a C₁₅ skeleton with a Chromane ring bearing a second aromatic ring B in position 2, 3 or 4. Various flavonoids are classified on the basis of chemical structure as follows:

1.7.1 Flavones

It contains a benzo-γ-pyrone ring with phenyl substitution at C-2 position of the pyrone ring. (Apigenin and Luteolin are examples of flavone).



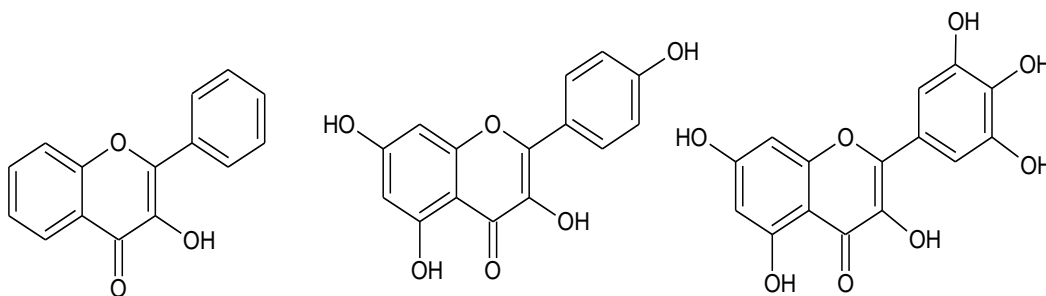
Flavone (Generalised structure)
(2-phenyl-chromen-4-one)

Apigenin

Luteolin

1.7.2 Flavonols

It is a 3-hydroxy derivative of flavone. The presence of a 3-OH group affects the UV absorption and color reaction. Quercetin, Kamferol and Myricetin are examples of flavonol.



Flavonol (Generalised structure)

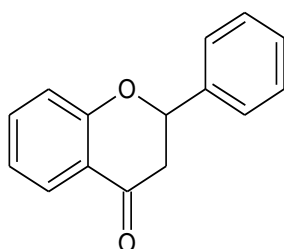
3-hydroxy-2-phenyl-4H-1-benzopyran-4-one

Kaempferol

Myricetin

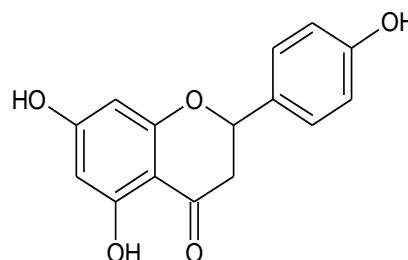
1.7.3 Flavanones

Flavanone are colorless compounds and are rather neglected group of flavonoids .they are characteristic o by absence of double bond at 2,3-position .It means that they are 2,3-dihydroderivative of flavone . Naringenin is an example of Flavanone.



Flavanone (Generalised structure)

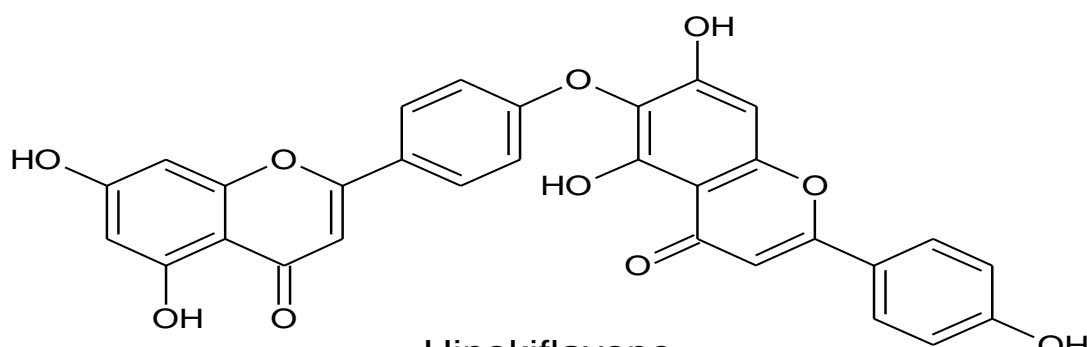
2-phenyl-2,3-dihydro-4H-1-benzopyran-4-one



Naringenin

1.7.4 Bioflavonoids

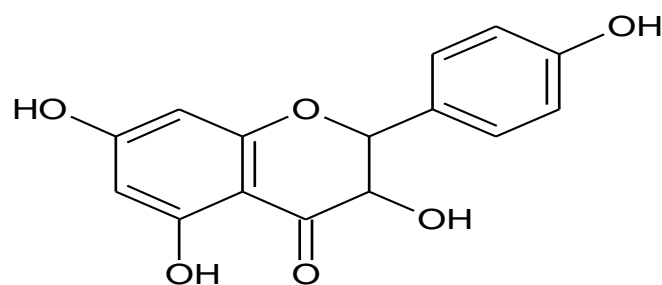
There are dimers of the various flavonoids. For example hinokiflavone it is a characteristics of gymnosperms .Bond is formed between very reactive C-6 and C-8 position.



Hinokiflavone

1.7.5 Dihydroflavonols

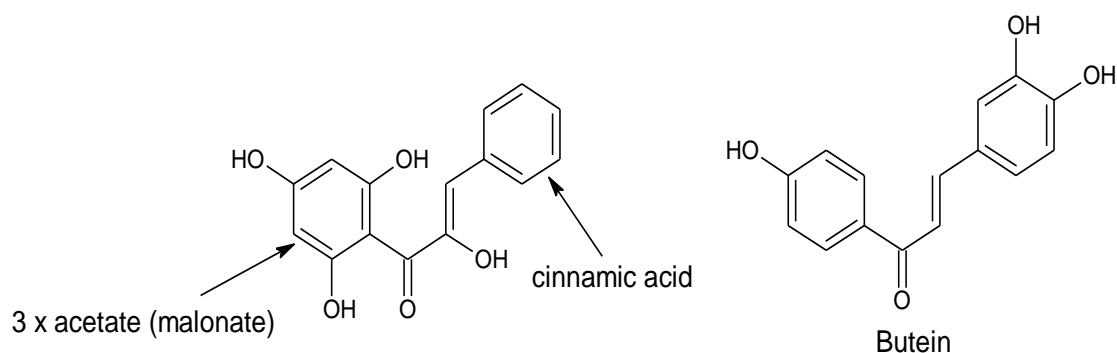
It is 3-hydroxy derivative of flavanone. Dihydroxykamferol is an example of dihydroflavonols.



Dihydroxykaemperol

1.7.6 Chalcones

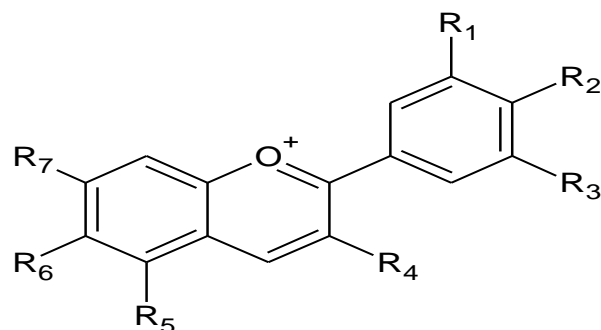
These do not contain γ -pyrone ring and hence are open chain flavonoids in which two aromatic ring are joined by three carbon α , β – unsaturated carbonyl system. Chalcone is one derived from three acetates and cinnamic acid. Butein is an example of chalcone, found in Free State in the heartwood of acacia.



1.7.7 Anthocyanins

These are water soluble vacuolar pigments that may appear red, purple or blue in color depending to pH. These consist of flavylium ion, the basic structure of which is given here. As shown, there are 7

different side groups on the flavyium ion. These side groups can be hydrogen atom, hydroxide or methoxy group.



Anthocyanins

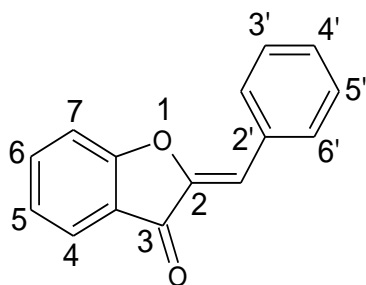
The most frequent combination of side groups and their names are shown in table (3).

Table (3): Most frequent combination of side groups and their names

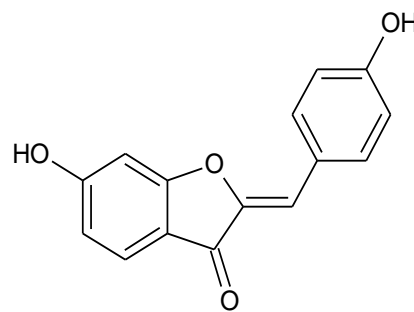
Anthocyanidin	R1	R2	R3	R4	R5	R6	R7	Main Color
Apigeninidin	H	OH	H	H	OH	H	OH	Orange
Aurantidin	H	OH	H	OH	OH	OH	OH	Orange
Cyanidin	OH	OH	H	OH	OH	H	OH	Magenta
Delphinidin	OH	OH	OH	OH	OH	H	OH	Purple-blue
Hirsutidin	OCH ₃	OH	OCH ₃	OH	OH	H	OCH ₃	Bluish - red

1.7.8 Aurones

In a few cases, the six-membered heterocyclic ring C occurs in an isomeric open form or is replaced by a five membered furan-nose ring. There are formed from chalcones by aerial or enzymatic oxidation and are deep yellow in color. Hispidol is an example of aurones.



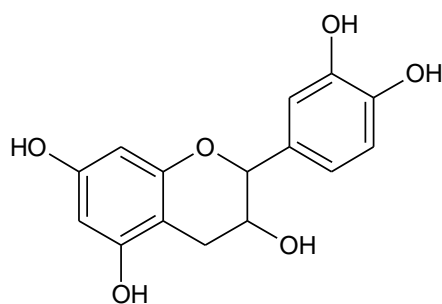
(2Z)-2-benzylidene-1-benzofuran-3(2H)-one



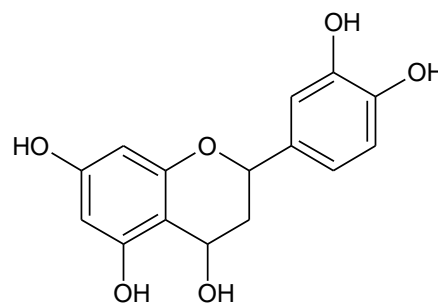
Hispidol

1.7.9 Flavanols

Flavanols contain hydroxyl substitution at C-3 position (flavan-3-ol) or C-3 and 4 positions (flavan-3,4-diol). Catechin and leucocyanidin are their examples respectively.



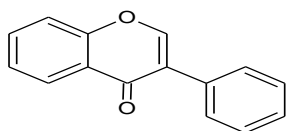
Catechin



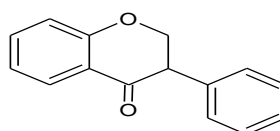
Leucocyanidin

1.7.10 Isoflavonones

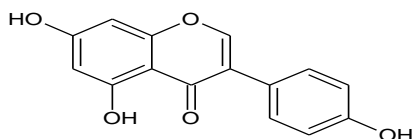
Isoflavonones contain benzo- γ -pyrone ring with phenyl substitution at C-3 position of the pyrone ring and isoflavanone is dihydroderivative of isoflavanone. Genistein and ferreranol are examples of isoflavone and isoflavanone respectively¹.



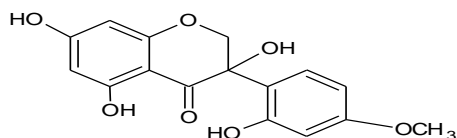
Isoflavonone
(3-phenyl-4*H*-1-benzopyran-4-one)



Isoflavanone
(3-phenyl-2,3-dihydro-4*H*-1-benzopyran-4-one)



Genistein
(5,7-dihydroxy-3-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one)



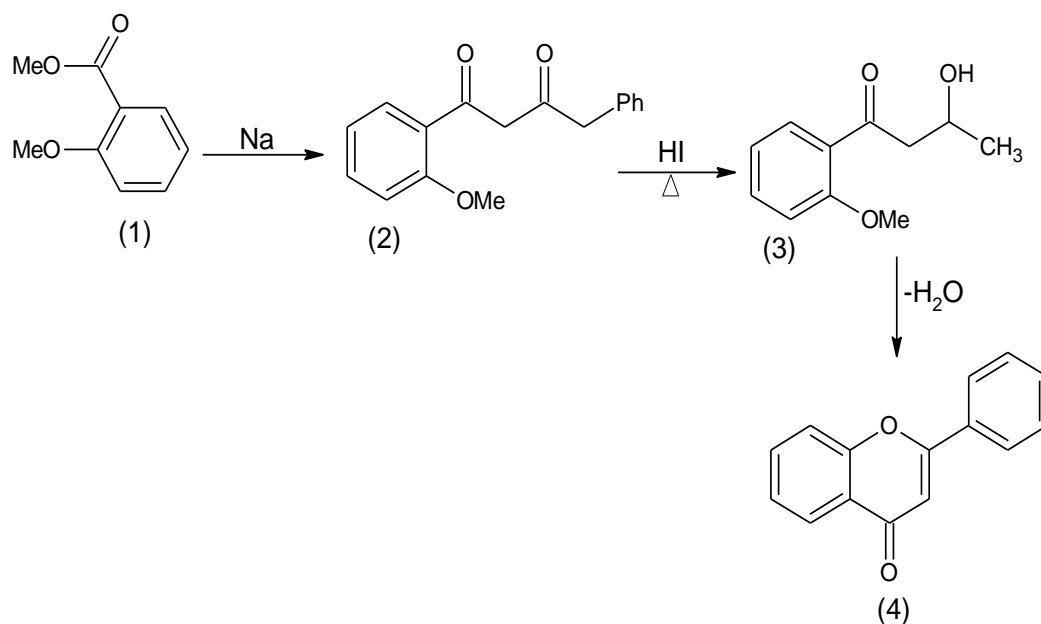
Ferreirinol
(3,5,7-trihydroxy-3-(2-hydroxy-4-methoxyphenyl)-2,3-dihydro-4*H*-1-benzopyran-4-one)

1.8 Synthesis of flavonoids

The interest in the biological properties of flavones has resulted in intense synthetic efforts towards the synthesis of various flavones. There are a number of methods reported for the synthesis of flavones¹.

1.8.1 The Von-Konstanecki method

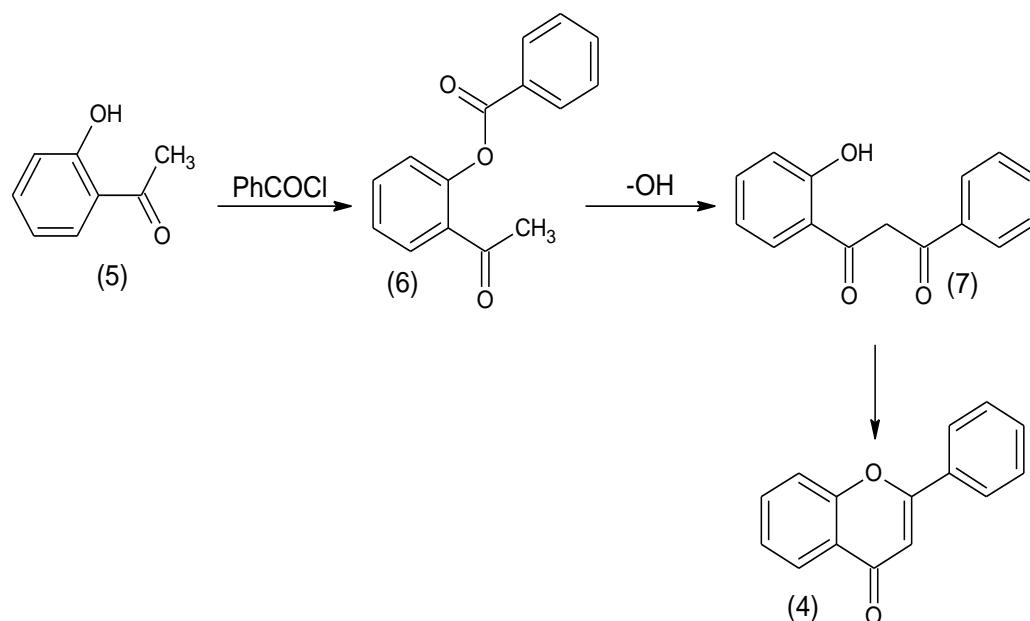
This is a general method for synthesizing flavones which involves a reaction of *o*-methoxybenzoate (1) and acetophenone in the presence of sodium to form (2) as shown in Scheme 1.1. The reaction occurs via the Claisen condensation. This is followed by treatment of (2) with an acid to form compound (3) followed by elimination of water in order to form the flavone (4)¹⁴.



Scheme 1.1 : The Von-Konstanecki method

1.8.2 The Baker-Venkataraman method

The Baker-Venkataraman approach would be the most convenient route to the synthesis of flavone as shown in Scheme 1.2. In Baker-Venkataraman reaction, 2-hydroxyacetophenone (5) was converted to ester (6), which then underwent rearrangement by intramolecular Claisen condensation in the presence of potassium hydroxide and pyridine to afford 1,3-diketone (7). Compound (7) was then cyclised to flavone (3) under rather harsh conditions either by treatment with concentrated sulfuric acid or heating with glacial acetic acid¹⁵.



Scheme 1.2: The Baker-Venkataraman method

1.9 Isolation of flavonoids

The analysis of flavonoids and their conjugates is one of the most important areas in the field of instrumental analytical methods, helping to solve problems in biological and medical sciences. Different methods of isolation of the natural products may be applied, and the utilization of various strategies is dependent on the origin of the biological material from which the target natural products are to be extracted (plant or animal tissue or body fluids). In the case of polyphenolic compounds, it is often important to initially determine whether the researchers are interested in the identification of individual components present in a mixture of target compounds or whether they would like to estimate the total amount of phenolic compounds in the biological material investigated. This second approach most often takes place during the nutritional studies on different foods or fodders, mainly of plant origin¹.

The presence of carbohydrates and/or lipophylic substances may influence the profile of the qualitative and quantitative composition of flavonoids and their derivatives in the obtained extracts. One has to

consider the above-mentioned selection of the methods for sample preparation and extraction, and in many cases additional cleaning based on solid-phase extraction (SPE) of the extracted samples is required^{1,42}.

1.9.1. Preparation of plant tissue for flavonoid analysis

The utilization of dried plant material for extraction may cause a substantial decrease in the yield of flavonoid conjugates. Acylated flavonoid glycosides are especially labile at elevated temperatures and are frequently thermally degraded during the process of drying plant tissues. This is important during the profiling of this class of natural products in research directed toward the investigation of their physiological and biochemical roles in plants under the influence of environmental factors, or in studies of genetically modified plants for the elucidation of changes in metabolic pathways. Free flavonoid aglycones exuded by plant tissues (leaf or root) may be washed from the surface with nonpolar solvents, such as methylene chloride, ethyl ether, or ethyl acetate. However, more polar glycosidic conjugates dissolve in polar solvents (methanol and ethanol), and these organic solvents are applied for extraction procedures in Soxhlet apparatus. Mixtures of alcohol and water in different ratios are applied for the extraction of flavonoids and their conjugates from solid biological material (plant or animal tissues and different food products). The extraction efficiency may be enhanced by the application of ultrasonication⁴³ or pressurized liquid extraction (PLE), a procedure performed at elevated temperature ranging from 60°C to 200°C⁴³. Supercritical fluid extraction with carbon dioxide also may be used, but procedures have to be carefully adjusted because of the possibility of thermal degradation of the flavonoid derivatives. In many cases, further purification and/or preconcentration of the target compound fraction is necessary. In these cases, liquid–liquid extraction (LLE) or (SPE) are most commonly used. For estimation of the extraction yield it is

necessary to spike biological materials with proper internal standards. Most suitable are compounds structurally similar to the studied analytes but not present in the sample. Compounds labeled with stable isotopes (^{13}C) are useful when mass spectrometric detection is applied. In the case of the extraction of flavonoids from biological materials, different classes of phenolic compounds are often added. On the other hand, quantitative analysis of consecutive components of the analyzed flavonoid mixture needs reference standard compounds necessary for preparation of calibration curves essential for a precise quantification. The choice of the extraction procedure for obtaining flavonoid conjugates from biological material is very important and depends on the goals of the conducted research. The evaluation of the spatial distribution of target compounds on the organ, tissue, cellular or even sub cellular level is of special interest in some projects. In these situations, the amount of biological material for the isolation of natural products may be extremely small, and the application of microextraction techniques is necessary⁴⁴. In many cases, it is necessary to avoid the chemical and/or enzymatic degradation of the metabolites. This is of special importance in the profiling of flavonoids glycosides in research directed toward plant functional genomics or during physiological and biochemical studies that need information about all classes of flavonoids conjugates present, even the thermally labile acylated derivatives. On the other hand, in the phytochemical analysis of plant species or phytopharmaceutical studies of plant material, the repeatable isolation of all biologically active flavonoid aglycones with a good yield is more important. In these cases, more drastic extraction conditions are acceptable. Excellent reviews have been published on isolation strategies for the determination of active phenols in plants tissue or food and foodstuff^{45,46}. However, the temperature conditions during the extraction robust multistep chromatographic

methods are necessary for the isolation of individual components from plant extracts containing new uncharacterized compounds. Various stationary phases are used in column chromatography, including polyamide, Sephadex LH-20, and different types of silica gels (normal and reversed phase with chemically bonded functional groups). The proper choice of solvent systems is necessary, often requiring the application of gradients of more polar (normal phases) or more hydrophobic solvents (reverse phases), together with the above-mentioned chromatographic supports in different chromatography systems. The sequence and kind of separation methods used depends on the composition of the sample and the experience of the researcher. However, minor flavonoid components are difficult to obtain as pure compounds. In cases of analysis of samples containing a number of compounds present in small amounts, the application of an analytical chromatographic systems enhanced by proper detectors (UV, NMR, and/or MS) gives spectrometric information sufficient for establishing the structure of minor target components. When liquid chromatography is used for separation of compounds, multiple detector systems are available (UV diode array detector, mass spectrometers, and nuclear magnetic resonance spectrometer). It is possible to achieve complete structural information about isomeric flavonoids and their conjugates in this way.

1.10 Structural characterization of flavonoids

1.10.1 UV spectroscopy of flavonoids

Generally, flavonoids produce two bands in UV spectrum: band I due to the cinnamoyl part (around 350 nm) and band II due to the benzoyl (around 250 nm) as shown in table (4)⁴⁷.

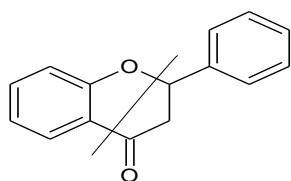


Table (1. 4) : U.V maximum absorbance for different flavonoids classes

Name	Band II (nm)	Band I (nm)
Flavone	250 – 280	300 – 350
Flavonol	250 – 265	350 – 380
Flavanone	270 – 295	300 - 330
Flavanol	290	320
Isoflavone	245 – 270	300 - 340
Chalcone	220 – 270	340 - 390
Anthocyanin	270 – 280	460 - 550

The methanol spectra of flavones and flavonols, exhibits two major absorption peak in the region, 240-400 nm. Band I at 300-380 nm appears due to cinnamoyl function and band II at 240-280 nm for benzoyl function present in this structure.

The positions of hydroxyl groups at different positions can be identified with the help of shift reagents like, CH_3ONa , CH_3COONa , AlCl_3 , H_3BO_3 , etc. these reagents cause shift in λ_{max} ⁴⁸.

1.10.2 Nuclear magnetic resonance

NMR is a well-established and the most commonly used method for natural product structure analysis. The studies of flavonoid

Structures using ^1H -NMR were initiated in 1960s⁴⁹ and along with ^{13}C -NMR have become the method of choice for the structure elucidation of these compounds. The chemical shifts and multiplicity of signals corresponding to particular atoms and their coupling with other atoms within the molecule allow for easy identification of the aglycone structure, the pattern of glycosylation, and the identity of the sugar moieties present.

1.10.3 Mass spectroscopy

MS is a very sensitive analytical method used to identify flavonoid conjugates or to perform partial structural characterization using microgram amounts of sample⁵⁰. Indeed, significant structural data can be obtained from less than 1 mg of the analyzed compound when different MS techniques are used in combination with chemical derivatization of the characterized compounds⁵¹. Flavonoid glycosides are thermally labile compounds and the evaporation without decomposition of the analyte is impossible, even in the ion source of a MS, where high vacuum exists (about 3×10^{-5} torr). In this situation, soft ionization methods need to be applied for the analysis of this group of compounds, and the analyte molecules are ionized without evaporation in high vacuum (FAB or LSIMS, MALDI) or under atmospheric pressure (ESI, APCI). From normal mass spectra, information can be obtained about the molecular weight of the whole conjugate, the size of the sugar moieties attached to the aglycone, and the molecular weight of the aglycone^{51,52}.

1.11 Biological activity of flavonoids

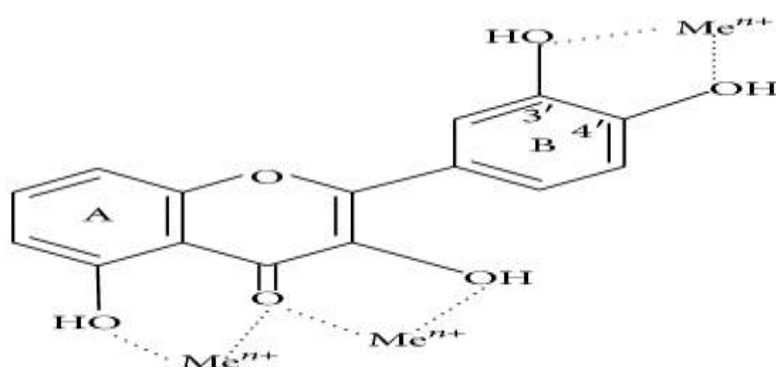
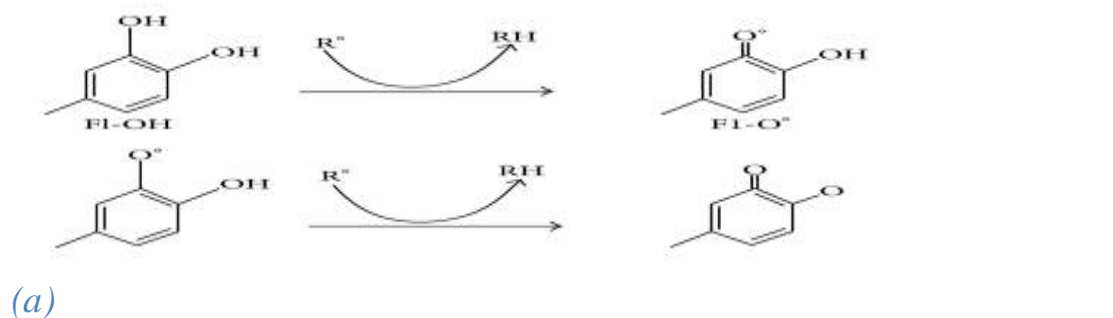
1.11.1 Antioxidant activity

Flavonoids possess many biochemical properties, but the best described property of almost every group of flavonoids is their capacity to act as antioxidants. The antioxidant activity of flavonoids depends upon the arrangement of functional groups about the nuclear structure. The configuration, substitution, and total number of hydroxyl groups substantially influence several mechanisms of antioxidant activity such as radical scavenging and metal ion chelation ability^{4,53}. The B ring hydroxyl configuration is the most significant determinant of scavenging of ROS and RNS because it donates hydrogen and an electron to hydroxyl, peroxy, and peroxyxynitrite radicals, stabilizing them and giving rise to a relatively stable flavonoids radical⁵⁴.

Mechanisms of antioxidant action can include (1) suppression of ROS formation either by inhibition of enzymes or by chelating trace elements involved in free radical generation; (2) scavenging ROS; and (3) upregulation or protection of antioxidant defenses^{55,56}. Flavonoid action involves most of the mechanisms mentioned above. Some of the effects mediated by them may be the combined result of radical scavenging activity and the interaction with enzyme functions. Flavonoids inhibit the enzymes involved in ROS generation, that is, microsomal monooxygenase, glutathione S-transferase, mitochondrial succinoxidase, NADH oxidase, and so forth⁵⁷.

Lipid peroxidation is a common consequence of oxidative stress. Flavonoids protect lipids against oxidative damage by various mechanisms^{5,26}. Free metal ions enhance ROS formation by the reduction of hydrogen peroxide with generation of the highly reactive hydroxyl radical. Due to their lower redox potentials flavonoids (Fl-OH) are thermodynamically able to reduce highly oxidizing free radicals (redox potentials in the range 2.13–1.0 V) such as superoxide, peroxy, alkoxy, and hydroxyl radicals by hydrogen atom donation (Figure [1\(a\)](#)). Because of their capacity to chelate metal ions (iron, copper, etc.), flavonoids also inhibit free radical generation^{56,58}. Quercetin in particular is known for its iron-chelating and iron-stabilizing properties. Trace metals bind at

specific positions of different rings of flavonoid structures⁵⁹. The binding sites are shown in Figure 1(b).



(b)
Figure 1: (a) Scavenging of ROS (R°) by flavonoids (Fl-OH) and (b) binding sites for trace metals where indicates metal ions.

A 3',4'-catechol structure in the B ring firmly enhances inhibition of lipid peroxidation. This trait of flavonoids makes them most effective scavengers of peroxy, superoxide, and peroxynitrite radicals⁴. Epicatechin and rutin are strong radical scavengers and inhibitors of lipid peroxidation *in vitro*⁶⁰. Oxidation on the B ring of flavonoids having catechol group afford fairly stable ortho-semiquinone radical which is strong scavengers. Flavones lacking catechol system on oxidation lead to formation of unstable radicals that exhibit weak scavenging potential⁶¹. The literature shows that flavonoids having an unsaturated 2-3 bond in conjugation with a 4-oxo function are more potent antioxidants than the

flavonoids lacking one or both features. Conjugation between the A and B rings allows a resonance effect of the aromatic nucleus that provides stability to the flavonoid radical. Free radical scavenging by flavonoids is potentiated by the presence of both the elements besides other structural features⁶².

The flavonoid heterocycle contributes to antioxidant activity by permitting conjugation between the aromatic rings in presence of a free 3-OH. Removal of a 3-OH annuls coplanarity and conjugation which compromises scavenging ability⁶³. It is proposed that B ring OH groups form hydrogen bonds with the 3-OH, aligning the B ring with the heterocycle and A ring. Due to this intramolecular hydrogen bonding, the influence of a 3-OH is enhanced by the presence of a 3',4'-catechol, elucidating the potent antioxidant activity of flavan-3-ols that possess the latter feature. Generally O-methylation of hydroxyl groups of flavonoids decreases their radical scavenging capacity⁶⁴.

Occurrence, position, structure, and total number of sugar moieties in flavonoid (flavonoids glycosides) play an important role in antioxidant activity. Aglycones are more potent antioxidants than their corresponding glycosides. There are reports that the antioxidant properties of flavonol glycosides from tea declined as the number of glycosidic moieties increased⁶⁵. Though glycosides are usually weaker antioxidants than aglycones, bioavailability is sometimes enhanced by a glucose moiety. In the diet, flavonoid glycosidic moieties occur most frequently at the 3- or 7-position⁶⁵. Increasing degree of polymerization enhances the effectiveness of procyanidins against a variety of radical species. Procyanidin dimers and trimers are more effective than monomeric flavonoids against superoxide anion. Tetramers exhibit greater activity against peroxynitrite and superoxide mediated oxidation than trimers,

while heptamers and hexamers demonstrate significantly greater superoxide scavenging properties than trimers and tetramers⁶⁶.

1.11.2 Hepatoprotective activity

Several flavonoids such as catechin, apigenin, quercetin, naringenin, rutin, and venoruton are reported for their hepatoprotective activities⁶⁷. Different chronic diseases such as diabetes may lead to development of hepatic clinical manifestations.

Anthocyanins have drawn increasing attention because of their preventive effect against various diseases. Zhu et al. [68] demonstrated that anthocyanin cyanidin-3-O- β -glucoside (C3G) increases hepatic Gclc expression. Increased Gclc expression results in a decrease in hepatic ROS levels and proapoptotic signaling. Furthermore, C3G treatment lowers hepatic lipid peroxidation, inhibits the release of proinflammatory cytokines, and protects against the development of hepatic steatosis⁶⁸.

Silymarin is a flavonoids having three structural components silibinin, silydianine, and silychristine extracted from the seeds and fruit of milk thistle (*Silybum marianum*). Silymarin has been reported to stimulate enzymatic activity of DNA-dependent RNA polymerase 1 and subsequent biosynthesis of RNA and protein, resulting in DNA biosynthesis and cell proliferation leading to liver regeneration only in damaged livers⁶⁹. The pharmacological properties of silymarin involve the regulation of cell membrane permeability and integrity, inhibition of leukotriene, ROS scavenging, suppression of NF- κ B activity, depression of protein kinases, and collagen production⁷⁰. Silymarin has clinical applications in the treatment of cirrhosis, ischemic injury, and toxic hepatitis induced by various toxins such as acetaminophen, and toxic mushroom^{71,72}.

Hepatoprotective activities were observed in flavonoids isolated from *Laggera alata* against carbon-tetrachloride (CCl₄-) induced injury in

primary cultured neonatal rat hepatocytes and in rats with hepatic damage. Flavonoids at a concentration range of 1–100 µg/mL improved cell viability and inhibited cellular leakage of hepatocyte aspartate aminotransferase (AST) and alanine aminotransferase (ALT) caused by CCl₄⁷². Similarly in an in vivo experiment flavonoids at 50, 100, and 200 mg/kg oral doses significantly reduced the levels of AST, ALT, total protein, and albumin in serum and the hydroxyproline and sialic acid levels in liver. Histopathological examinations also revealed the improvement in damaged liver with the treatment of flavonoid⁷³.

Several clinical investigations have shown the efficacy and safety of flavonoids in the treatment of hepatobiliary dysfunction and digestive complaints, such as sensation of fullness, loss of appetite, nausea, and abdominal pain. Equisetum arvense flavonoids as well as hirustrin and avicularin isolated from some other sources are reported to provide protection against chemically induced hepatotoxicity in HepG2 cells^{73,74}.

1.11.3 Antimicrobial activity

Flavonoids are known to be synthesized by plants in response to microbial infection; thus it should not be surprising that they have been found in vitro to be effective antimicrobial substances against a wide array of microorganisms. Flavonoid rich plant extracts from different species have been reported to possess antibacterial activity^{75,76}. Several flavonoids including apigenin, galangin, flavone and flavonol glycosides, isoflavones, flavanones, and chalcones have been shown to possess potent antibacterial activity⁷⁷.

Antibacterial flavonoids might be having multiple cellular targets, rather than one specific site of action. One of their molecular actions is to form complex with proteins through nonspecific forces such as hydrogen

bonding and hydrophobic effects, as well as by covalent bond formation. Thus, their mode of antimicrobial action may be related to their ability to inactivate microbial adhesins, enzymes, cell envelope transport proteins, and so forth. Lipophilic flavonoids may also disrupt microbial membranes^{78,79}.

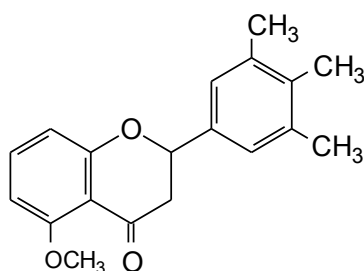
Catechins, the most reduced form of the C3 unit in flavonoid compounds, have been extensively researched due to their antimicrobial activity. These compounds are reported for their in vitro antibacterial activity against *Vibrio cholerae*, *Streptococcus mutans*, *Shigella*, and other bacteria^{80,81}. The catechins have been shown to inactivate cholera toxin in *Vibrio cholera* and inhibit isolated bacterial glucosyl transferase in *S. mutans*, probably due to complexing activities^{80,82}. Robinetin, myricetin, and (–)-epigallocatechin are known to inhibit DNA synthesis in *Proteus vulgaris*. Mori et al.⁸³ suggested that the B ring of the flavonoids may interchelate or form hydrogen bond with the stacking of nucleic acid bases and further lead to inhibition of DNA and RNA synthesis in bacteria. Another study demonstrated inhibitory activity of quercetin, apigenin, and 3,6,7,3',4'-pentahydroxyflavone against *Escherichia coli* DNA gyrase⁸⁴.

Naringenin and sophoraflavanone G have intensive antibacterial activity against methicilline resistant *Staphylococcus aureus* (MRSA) and streptococci. An alteration of membrane fluidity in hydrophilic and hydrophobic regions may be attributed to this effect which suggests that these flavonoids might reduce the fluidity of outer and inner layers of membranes⁸⁵. The correlation between antibacterial activity and membrane interference supports the theory that flavonoids may demonstrate antibacterial activity by reducing membrane fluidity of bacterial cells. The 5,7-dihydroxylation of the A ring and 2',4'-or 2',6'-dihydroxylation of the B ring in the flavanone structure is important for

anti-MRSA activity⁸⁶. A hydroxyl group at position 5 in flavanones and flavones is important for their activity against MRSA. Substitution with C8 and C10 chains may also enhance the antistaphylococcal activity of flavonoids belonging to the flavan-3-ol class⁸⁷. Osawa et al. have shown that 5-hydroxyflavanones and 5-hydroxyisoflavanones with one, two, or three additional hydroxyl groups at the 7, 2' and 4' positions inhibited the growth of *S. mutans* and *Streptococcus sobrinus*⁸⁸.

Haraguchi and colleagues⁸⁶ studied antibacterial activity of two flavonoids, licochalcones A and C, isolated from the roots of *Glycyrrhiza inflata* against *S. aureus* and *Micrococcus luteus*. They observed that licochalcone A inhibited incorporation of radioactive precursors into macromolecules (DNA, RNA, and protein). This activity was similar to the mode of action of antibiotics inhibiting respiratory chain, since energy is required for active uptake of various metabolites as well as for biosynthesis of macromolecules. After further studies it was suggested that the inhibition site of these flavonoids was between CoQ and cytochrome in the bacterial respiratory electron transport chain⁸⁶. There are many examples that lend support to the prowess of phytoconstituents derived from edible and medicinal plants as potent antibacterial agents^{89,90}.

A flavanone: 5-methoxy-3', 4', 5'-trimethylflavanone(8) was isolated from the leaves of Sudanese *Albizia amara* and its structure was deduced on the basis of its spectral data (IR, UV, ¹HNMR and MS)⁹¹.

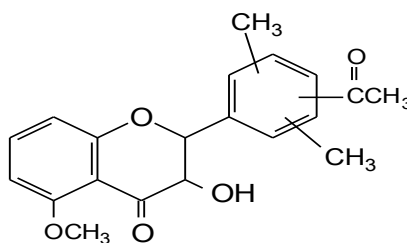


(8)

5-methoxy-3', 4', 5'-trimethylflavanone

The isolate was evaluated for its antimicrobial activity against six standard human pathogens : two Gram positive (*Staphylococcus aureus* and *Bacillus subtilis*) , two Gram negative (*Pseudomonas aeruginosa* and *Escherichia coli*) bacteria and two fungal species (*Aspergillus niger*, *Candida albicans*) and promising results suggested that the flavanone is a plausible candidate for further optimization⁹¹.

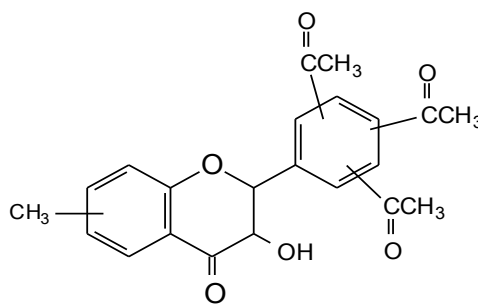
Also dihydroflavonol(9) was isolated⁹¹ from the same species by different chromatographic techniques and identified via a combination of spectral tools (IR, UV, ¹HNMR and mass spectroscopy).



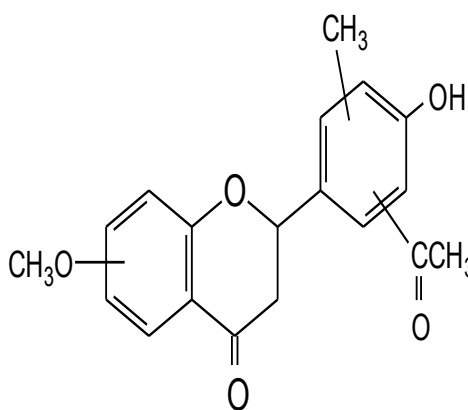
(9)

It was screened for its antimicrobial activity against six standard human pathogens (*Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Aspergillus niger*, *candida albicans*), and significant results were obtained.

A successive silica gel column chromatography followed by further purification via thin layer chromatography allowed for the isolation of two flavonoids (compounds 10 and 11) from fruits of *Vangueria infausta*. Identification of these compounds was based on extensive UV shifting reagents, IR, ¹HNMR and mass spectroscopy data. In well diffusion method, the chloroform fraction of *Vangueria infausta*, compounds I and II were evaluated for their antimicrobial activity⁹².



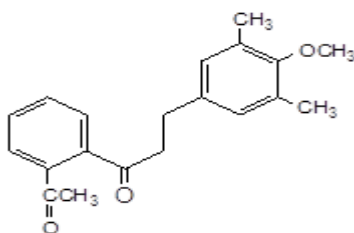
(10)



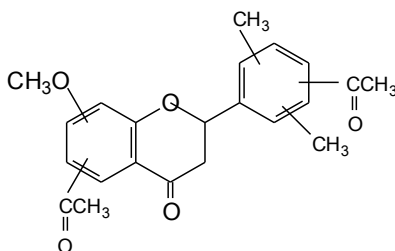
(11)

The chloroform fraction of *Vangueria infausta* did not show antibacterial activity, but it showed significant inhibitory activity against the fungi: *Candida albicans* and *Aspergillus niger*. Compounds 3 and 4 also showed antifungal activity. However, they did not reveal antibacterial activity⁹².

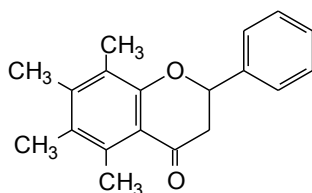
The following compounds were isolated⁹³ from the leaves of *Catharanthus roseus* and *Narissus brussonetii*. Compounds (12) and (13) were isolated from *Catharanthus roseus*, while compound (14) was isolated as yellow powder from *Narissus brussonetii* leaves by silica gel TLC using BAW(4:1:4) as solvent.



(12)



(13)



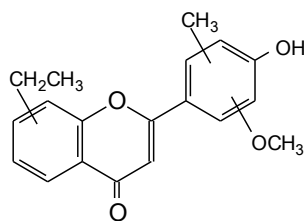
(14)

In cup plate agar diffusion assay ,the isolates were screened for antimicrobial activity against five standard human pathogens.

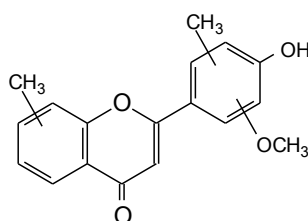
Compound 12 showed significant activity against the bacterial strain *Bacillus subtilis* and the fungal species *Candida albicans* , while compound 13 was active against *Escherichia coli* ,*Bacillus subtilis* and the fungus *Candida albicans*. However, compound 14 gave significant antifungal activity and partial antibacterial activity.

Phytochemical screening of the alcoholic extract of the roots of *Leptadenia heterophella* revealed the presence of tannins, saponins, terpenes, flavonoids and steroids. Alkaloid and glycosides were not detected. The crude alcoholic extract was fractionated by thin layer

chromatography. After the usual workup, the following compounds (15 and 16) were isolated. The structures of these isolates were elucidated by a combination of spectral tools (UV, IR, $^1\text{H NMR}$ and MS)⁹⁴.



(15)



(16)

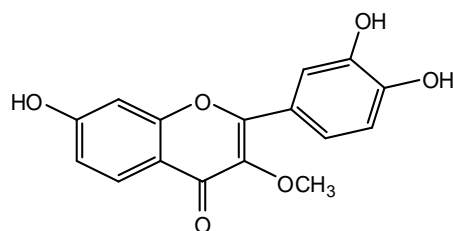
Different fractions of *Maytenas senegalensis* were evaluated⁹⁵ for their antimicrobial activity using the cup plate agar diffusion method. The ethanolic and ethyl acetate fractions of *Maytenas senegalensis* showed activity against all test organisms. The n-butanol fraction was active against all test organisms except *Escherichia coli* and the fungus *Aspergillus niger*. However, the chloroform extract showed partial activity against the bacterial strains but it did not show any antifungal activity (Table 3.1).

Table (3.1) : Antibacterial activity of *Maytenas senegalensis* extracts

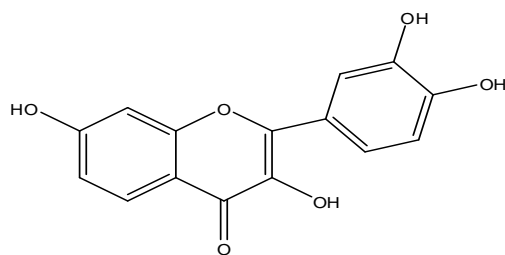
:M.D.I.Z (mm)

Extract	Conc.(mg/ml)	Ec	Pa	Sa	Bs	Ca	An
Ethanol	100	13	15	16	14	15	14
Ethyl acetate	100	16	14	15	16	13	13
n-butanol	100	12	13	13	16	13	12
Chloroform	100	12	12	12	12	-	-

From the methanolic extract of the heartwood of, *acacia nilotica* var *nilotica*, two compounds (17 and 18) were isolated (7, 3', 4'-trihydroxy-3-methoxyflavone and compound II was a 7, 3', 4'-trihydroxyflavonol respectively). The compounds were purified by different chromatographic techniques and identified via spectroscopic tools: IR, UV, ¹HNMR and Mass spectroscopy. The isolated compounds were evaluated for their antimicrobial potential against Gram negative (*Escherichia coli* and *Pseudomonasa eruginos*) and Gram positive (*Bacillus subtilis*, *Bacillus cereus* & *Staphylococcus aureus*) bacteria. The two compounds showed varying biological activity. compounds (17) and (18) were active against both Gram positive and Gram negative bacteria⁹⁶.

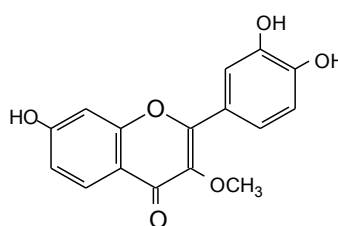


(17)



(18)

Phytochemical investigation of *Acacia nilotica* var *adstringens* heartwood led to the isolation of a flavone (19): 7,3',4'-trihydroxy-3-methoxyflavone from the methanolic extract. The crude extract was purified by a combination of chromatographic techniques (polyamide and Sephadex columns and paper chromatography). Structure of isolate was elucidated on the basis of extensive spectroscopic procedures including: IR, UV, ¹HNMR and MS. The isolated flavonoid was evaluated for its antibacterial potential against Gram negative (*Escherichia coli* and *Pseudomonasa eruginos*), Gram positive (*Bacillus subtilis*, *Bacillus cereus* and *Staphylococcus aureus*) bacteria. Compound I showed varying antibacterial responses. It showed high potency against Gram positive human pathogens: *Staphylococcus aureus* and *Bacillus subtilis*⁹⁶.



(19)

In well diffusion method, all fractions from *Albizia. Amararoots* showed inhibitory activity against *Streptococcus mutans* (Sm) and *Lacto bacillus* (Lb). The activity is expressed as less active, if the zone of inhibition is 9-12 mm, moderate: 15-16 mm and high if greater than 17 mm. The ethyl

acetate extract showed high activity on *Streptococcus mutans* (Sm) , while the n-butanol extract showed high activity on *Lacto bacillus* (Lb). The results of antimicrobial activity of the *A. amara* extracts against the microbial strains are depicted in Table 5⁹⁷.

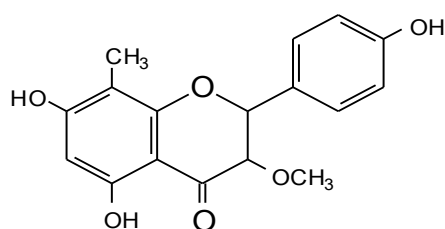
DMSO was used as solvent since it has no effect on the growth of any of the test microorganisms. Standard discs inhibited the growth of all the test microorganisms. . There has been an increasing effect on microbial growth inhibition with increasing concentration of the extracts However, the effects observed were less than those produced by the standard chemotherapeutic⁹⁷.

Table 3.2: Antibacterial activity of different fractions

Microorganism	Fractions	300µl	200 µl	100 µl
<i>Lacto bacillus</i> (LB)	PT	20	19	18
	CH	12	11	12
	EA	14	12	11
	BU	22	22	20
	ET	21	19	18
	AMP	25	25	25
	DMSO	-	-	-
<i>Streptococcus mutans</i> (SM)	PT	9	10	11
	CH	15	14	12
	EA	20	23	22
	BU	18	14	15
	ET	20	20	20
	AMP	25	25	25
	DMSO	-	-	-

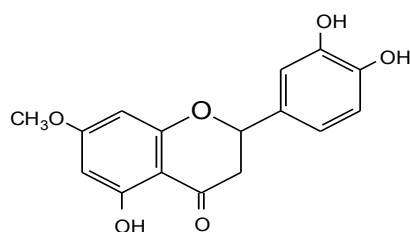
(ET = 0.09 , CH = 0.08, EA = 0.1 , P =0.03 , BU = 0.03) mg/ml , 100 μ l of sample + ml of DMSO.

Phytochemical screening of *Anogeissus leiocarpus* fruits indicated the presence of flavonoids, terpenes, tannins and saponins. Compound (20) was isolated from ethanolic extract by column and thin layer chromatography and its structure was established on the basis of its spectral data (IR, UV, NMR and MS). In *in vitro* studies, the isolated flavonoid gave promising antibacterial activity against *Escherichia coli* and moderate activity against *S. aureus*⁹⁸.



(20)

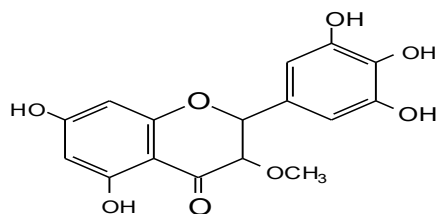
Adil reported⁹⁹ the isolation of a flavanone (21) (5,3',4'-trihydroxy-7-methoxyflavanone) from the Saudi material of *Cassia Italica*. The flavonoid was isolated from the ethyl acetate fraction by column chromatography. The structure was elucidated by sensitive analytical tools (UV, IR, ¹H NMR, ¹³C NMR, ¹H-¹H-COSY NMR and MS). The flavanone was evaluated, *in vivo*, for anti-inflammatory and anti-ulcer potential and significant results were obtained.



(21)

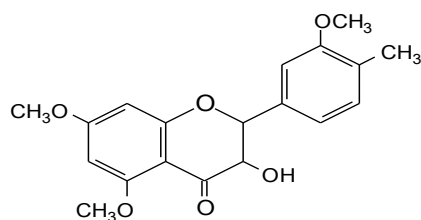
Phytochemical screening of the leaves of *Geigeria alata* revealed⁹⁸ the presence of flavonoids, tannins and alkaloids. Compound (22) was

isolated from ethanolic extract by column and thin layer chromatography and its structure was established on the basis of its spectral data (IR, UV, NMR and MS). Compound I and different extracts (ethanolic, chloroform, n- butanol and ethyl acetate) of *Geigeria alata* were screened for their antimicrobial activity against six standard human pathogens. The ethyl acetate fraction showed significant antimicrobial activity followed by the n-butanol fraction. However, the chloroform fraction exhibited moderate activity. Though, compound I showed significant antibacterial activity it did not afford any antifungal properties.



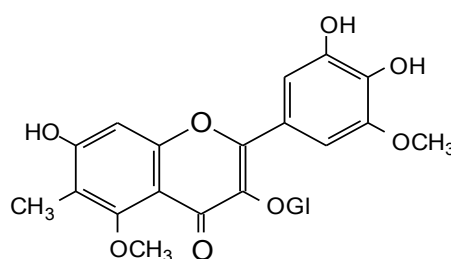
(22)

Amani¹⁰⁰ reported on the characterization and antimicrobial potency of a dihydroflavonol(23) isolated from the leaves of Sudanese *Tamarix nilotica*. The isolate was purified by different chromatographic techniques and identified via a combination of spectral tools(IR, UV, ¹HNMR and Mass spectroscopy). The isolated flavonoid, ethyl acetate and n-butanol fractions were evaluated(*in vitro*) for their antimicrobial potential against Gram negative (*Escherichia coli*, *Salomonella typhi*) and Gram positive (*Bacillus subtilis*, *Staphylococcus aureus*)bacteria and the fungi :*Candida albicans* and *Aspergillus niger* and promising results were obtained.The isolated dihydroflavonol seems to be a suitable candidate for future optimization.



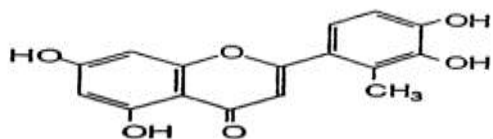
(23)

From the leaves of *Vitex doniana* (Sweet) a flavone (24) was isolated and characterized¹⁰¹. The isolate was purified by different chromatographic techniques and identified via a combination of spectral tools (IR, UV, ¹HNMR and Mass spectroscopy). The methanolic fraction of *Vitex doniana* was evaluated (*in vitro*) for its antimicrobial potential against Gram negative (*Escherichia coli*, *Salomonella typhimurium* and *Pseudomonasa eruginosa*) and Gram positive (*Bacillus subtilis*, *Bacillus aureus* and *Staphylococcus aureus*) bacteria and the fungus *Candidaalbicans*. Promising results were obtained. *In vitro* antioxidant assay for the methanolic extract was conducted. Evaluation of the antioxidant activity was carried out by measuring the capacity of the extract against stable DPPH radical. The extract showed significant antioxidant activity.



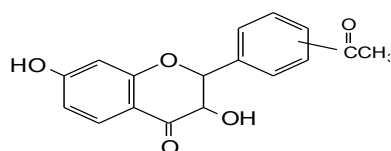
(24)

The crude ethanolic extract of *Croton Zambesicus* gave¹⁰² after paper chromatography a pure flavonoid (25) isolated as brown powder from ethanolic extract of the seeds of *CrotonZambesicus*.



(25)

Compound (26) was isolated as yellow powder from ethanolic extract of the seeds of *Coriandrum Stivum*. In cup plate agar diffusion assay, the crude extracts of *Croton Zambesicus*, *Coriandrum Stivum* and compounds (25 and 26) showed different antimicrobial responses against test organisms (Tables 3.3 and 3.4)¹⁰².



(26)

Table 3.3: The antimicrobial activity crude extractives of *Croton Zambesicus* and compounds (18) and (19)

Organism	Inhibition growth zone diameter (MIZD) 100 mg \ 100 ml		
	Crude extract	Compound I	Compound II
<i>Bacillus subtilis</i>	22	20	19

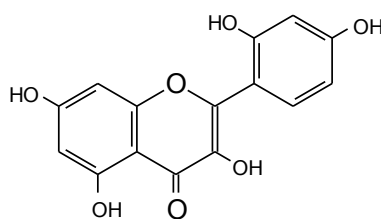
Staphylococcus aureus	21	20	17
Escherichia coli	22	19	18
Pseudomonas aeruginosa	22	20	18
Aspergillus niger	19	16	15
Condida albicans	16	15	14

Table 3.4: The antimicrobial activity crude extractives of *Coriandrum Stivum*

Organism	Crude extract
<i>Bacillus subtilis</i>	20
<i>Staphylococcus aureus</i>	20
<i>Escherichia coli</i>	19

<i>Pseudomonas aeruginosa</i>	22
<i>Aspergillus niger</i>	18
<i>Condida albacans</i>	20

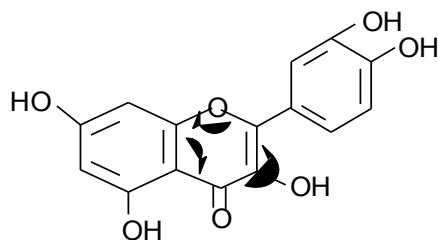
From the ethyl acetate extract⁹⁹ of the leaves *Cassia occidentalis* (Leguminosae) a flavonol: 5,7,2',4'-tetrahydroxyflavonol(27) was isolated. The structure was elucidated via a combination of spectral techniques (UV, IR, ¹H NMR and MS) and its biological potential was evaluated. The flavonol exhibited promising anti-inflammatory and anti-ulcer activity.



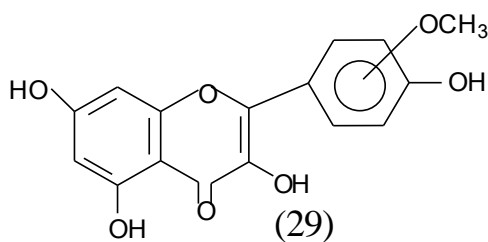
(27)

The antibacterial activity of the crude extract and pure flavonoids of *Withania somnifera* against five human pathogens was carried out¹⁰³. The cup-plate agar diffusion method was adopted with some minor modifications. The test organisms were: *Bacillus subtilis*, *Escherichia coli*, *Neisseria gonorrhoeae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The methanolic extract of *W. somnifera* showed moderate inhibition against *Escherichia coli*, *Pseudomonas aeruginosa* and

Staphylococcus aureus and weak inhibition against *Neisseria gonorrhoeae* and *Bacillus subtilis*. A flavonoid of *Withania somnifera* (28) exhibited a moderate inhibition against all five test organisms. Another flavonoid (29) showed weak inhibition against test organisms.



(28)



(29)

1.11.4 Anti-inflammatory activity

Inflammation is a normal biological process in response to tissue injury, microbial pathogen infection, and chemical irritation. Inflammation is initiated by migration of immune cells from blood vessels and release of mediators at the site of damage. This process is followed by recruitment of inflammatory cells, release of ROS, RNS, and proinflammatory cytokines to eliminate foreign pathogens, and repairing injured tissues. In general, normal inflammation is rapid and self-limiting, but aberrant resolution and prolonged inflammation cause various chronic disorders¹⁰⁴.

The immune system can be modified by diet, pharmacologic agents, environmental pollutants, and naturally occurring food chemicals. Certain

members of flavonoids significantly affect the function of the immune system and inflammatory cells¹⁰⁵. A number of flavonoids such as hesperidin, apigenin, luteolin, and quercetin are reported to possess anti-inflammatory and analgesic effects. Flavonoids may affect specifically the function of enzyme systems critically involved in the generation of inflammatory processes, especially tyrosine and serine-threonine protein kinases^{106,107}. The inhibition of kinases is due to the competitive binding of flavonoids with ATP at catalytic sites on the enzymes. These enzymes are involved in signal transduction and cell activation processes involving cells of the immune system. It has been reported that flavonoids are able to inhibit expression of isoforms of inducible nitric oxide synthase, cyclooxygenase, and lipoxygenase, which are responsible for the production of a great amount of nitric oxide, prostanoids, leukotrienes, and other mediators of the inflammatory process such as cytokines, chemokines, or adhesion molecules¹⁰⁸. Flavonoids also inhibit phosphodiesterases involved in cell activation. Much of the anti-inflammatory effect of flavonoid is on the biosynthesis of protein cytokines that mediate adhesion of circulating leukocytes to sites of injury. Certain flavonoids are potent inhibitors of the production of prostaglandins, a group of powerful proinflammatory signaling molecules¹⁰⁹.

Reversal of the carrageenan induced inflammatory changes has been observed with silymarin treatment. It has been found that quercetin inhibit mitogen stimulated immunoglobulin secretion of IgG, IgM, and IgA isotypes *in vitro*¹¹⁰. Several flavonoids are reported to inhibit platelet adhesion, aggregation, and secretion significantly at 1–10 mM concentration¹¹¹. The effect of flavonoid on platelets has been related to the inhibition of arachidonic acid metabolism by carbon monoxide¹¹². Alternatively, certain flavonoids are potent inhibitors of cyclic AMP

phosphodiesterase, and this may in part explain their ability to inhibit platelet function.

1.11.5 Anti-cancer activity

Dietary factors play an important role in the prevention of cancers. Fruits and vegetables having flavonoids have been reported as cancer chemopreventive agents¹¹³. Consumption of onions and/or apples, two major sources of the flavonol quercetin, is inversely associated with the incidence of cancer of the prostate, lung, stomach, and breast. In addition, moderate wine drinkers also seem to have a lower risk to develop cancer of the lung, endometrium, esophagus, stomach, and colon¹¹⁴. The critical relationship of fruit and vegetable intake and cancer prevention has been thoroughly documented. It has been suggested that major public health benefits could be achieved by substantially increasing consumption of these foods.¹¹⁵

Several mechanisms have been proposed for the effect of flavonoids on the initiation and promotion stages of the carcinogenicity including influences on development and hormonal activities.¹¹⁶ Major molecular mechanisms of action of flavonoids are given as follows:(1)downregulation of mutant protein,(2)cell cycle arrest,(3)tyrosine kinase inhibition,(4)inhibition of heat shock proteins,(5)estrogen receptor binding capacity,(6)inhibition of expression of Ras proteins.

Mutations of are among the most common genetic abnormalities in human cancers. The inhibition of expression of may lead to arrest the cancer cells in the G2-M phase of the cell cycle. Flavonoids are found to downregulate expression of mutant p53 protein to nearly undetectable levels in human breast cancer cell lines¹¹⁷. Tyrosine kinases are a family of proteins located in or near the cell membrane involved in the

transduction of growth factor signals to the nucleus. Their expression is thought to be involved in oncogenesis via an ability to override normal regulatory growth control. Drugs inhibiting tyrosine kinase activity are thought to be possible antitumor agents without the cytotoxic side effects seen with conventional chemotherapy. Quercetin was the first tyrosine kinase inhibiting compound tested in a human phase I trial¹¹⁸. Heat shock proteins form a complex with mutant p53, which allows tumor cells to bypass normal mechanisms of cell cycle arrest. Heat shock proteins also allow for improved cancer cell survival under different bodily stresses. Flavonoids are known to inhibit production of heat shock proteins in several malignant cell lines, including breast cancer, leukemia, and colon cancer¹¹⁷.

Recently it has been shown that the flavanol epigallocatechin-3-gallate inhibited fatty acid synthase (FAS) activity and lipogenesis in prostate cancer cells, an effect that is strongly associated with growth arrest and cell death^{114,119}. In contrast to most normal tissues expression of FAS is markedly increased in various human cancers. Upregulation of FAS occurs early in tumor development and is further enhanced in more advanced tumors¹²⁰.

Quercetin is known to produce cell cycle arrest in proliferating lymphoid cells. In addition to its antineoplastic activity, quercetin exerted growth-inhibitory effects on several malignant tumor cell lines in vitro. These included P-388 leukemia cells, gastric cancer cells (HGC-27, NUGC-2, NKN-7, and MKN-28), colon cancer cells (COLON 320 DM), human breast cancer cells, human squamous and gliosarcoma cells, and ovarian cancer cells¹¹⁷. Markaverich et al.¹²¹ proposed that tumor cell growth inhibition by quercetin may be due to its interaction with nuclear type II estrogen binding sites (EBS). It has been experimentally proved that

increased signal transduction in human breast cancer cells is markedly reduced by quercetin acting as an antiproliferative agent¹²² .

Barnes¹²³ has extensively reviewed the anticancer effects of genistein on in vitro and in vivo models. In an study to determine effects of isoflavones genistein, daidzein, and biochanin A on mammary carcinogenesis, genistein was found to suppress the development of chemically induced mammary cancer without reproductive or endocrinological toxicities. Neonatal administration of genistein (a flavonoid) exhibited a protective effect against the subsequent development of induced mammary cancer in rats¹²⁴ .Hesperidin, a flavanone glycoside, is known to inhibit azoxymethanol induced colon and mammary cancers in rats¹²⁵ . The anticancer properties of flavonoids contained in citrus fruits have been reviewed by Carroll et al.¹²⁶ . Several flavonols, flavones, flavanones, and the isoflavones biochanin A are reported to have potent antimutagenic activity¹²⁷ . A carbonyl function at C-4 of the flavone nucleus was found to be essential for their activity. Flavone-8-acetic acid has also been shown to have antitumor effects¹²⁸ . In earlier studies ellagic acid, robinetin, quercetin, and myricetin have been shown to inhibit the tumorigenicity of BP-7, 8-diol-9, and 10-epoxide-2 on mouse skin¹²⁹ .

Higher consumption of phytoestrogens, including isoflavones and other flavonoids, has been shown to provide protection against prostate cancer risk¹³⁰ . It is well known that due to oxidative stress cancer initiation may take place and thus potent antioxidants show potential to combat progression of carcinogenesis. Potential of antioxidant as an anticancer agent depends on its competence as an oxygen radical inactivator and inhibitor¹³¹ . Therefore diets rich in radical scavengers would diminish the cancer-promoting action of some radicals¹³² .

Aim of this study

This study was designed to fulfill the following goals:

- Extraction of flavonoids from the target plants.
- Isolation of flavonoids via chromatographic techniques.
- Elucidation of structures of isolates.
- Screening for antimicrobial potential.

2. Material and Methods

2.1 Materials

2.1.1 Plant material

The stem bark of *Cordia sinensis* and *Acacia oerofota* were collected from Arrahad abudakana, North Kordofan, western Sudan in October 2014. The plants were authenticated by the direct comparison with herbarium samples.

2.1.2 Solvents

All Solvents used are of analytical grade. Methanol HPLC grade was used for spectroscopic purposes (BDH, England).

2.1.3 Chromatographic Materials

-Sheets of Whatman paper (No. 1 and No. 3 mm- 46 x 57 cm) from whatman Ltd. Kent, England.

-Glass jars, 10 x 20 x 24 cm, fitted with covers, were used for chromatographic fractionation.

2.1.4 Equipments

The ultra violet lamp used in visualizing TLC plates and paper chromatography was a multiband UV λ_{max} (254 / 365 nm) portable ultra violet, a product of Hanovia lamps (6 watt S/Y and L/W). Ultra violet absorption spectra were obtained in spectroscopic Methanol on UV - visible spectrophotometer (Shimadzu).

2.2 Methods

2.2.1 Preparations of reagents for phytochemical screening.

2.2.1.2 Flavonoid and phenolic test reagents

- Aluminium chloride solution

(1 g) of aluminum chloride was dissolved in 100 ml methanol

- Potassium hydroxide solution

(1g) of potassium hydroxide was dissolved in 100 ml distilled water.

-Ferric chloride solution

(1 g) of ferric chloride was dissolved in 100 ml methanol.

2.2.1.3 Alkaloid test reagents

Maeyer reagent

- Mercuric chloride solution: 1.36 g in 60 ml. distilled water.

- Potassium iodide solution: 5 g in 10 ml. distilled water

The two solutions were combined and then diluted with distilled water up to 100 ml.

-Wagner reagent

(1.27) g iodine and 2 g of potassium iodide in 100 ml distilled water.

2.2.2 Phytochemicalscreening

(100 g) of powdered shade - dried plant material were extracted with 80% aqueous methanol (soxhelt) until exhaustion. This prepared extract (PE) was used for phytochemical screening.

The prepared extract (PE) was used for following tests:

2.2.2.1 Test for unsaturated sterols and triterpenes

10 ml of the (PE) was evaporated to dryness on a water bath, and the cooled residue was stirred with petroleum ether to remove most of the coloring materials. The residue was then extracted with 10 ml chloroform. The chlorform solution was dehydrated over sodium sulphite anhydrous. 5 ml portion of the solution was mixed with 0.5 ml of acetic anhydride, followed by two drops of concentrated sulphuric acid. Two separate layers (green, red) were observed.

2.2.2.2 Test for flavonoids

(20 ml) of the (PE) was evaporated to dryness on water bath. The cooled residue was defatted with petroleum ether and then dissolved in 30 ml of 30% aqueous methanol and filtered. The filtrate was used for the following tests:

- To 3 ml. of filtrate a fragment of magnesium ribbon was added, shaken and then few drops of concentrated hydrochloric acid were added. Red colour was observed.
- To 3 ml. of the filtrate few drops of aluminium chloride solution were added. A dark yellow colour was formed.
- To 3 ml. of the filtrate few drops of potassium hydroxide solution were added. A dark yellow colour was observed.

2.2.2.3 Test for alkaloids

(10 ml) of the (PE) were evaporated to dryness on water bath and 5 ml of 0.2N hydrochloric acid were added and the solution was heated with stirring for minutes, then cooled and divided into two portions:

To one portion a few drops of Maeyer reagent were added. A white precipitated appeared, to the other portion few drops of Wagner reagent were added. A brown precipitate appeared.

2.2.2.4 Test for tannins

(10 ml) of (PE) was evaporated to dryness and the residue was extracted with n-hexane and then filtrated. The insoluble residue was stirred with n-hexane and 10 ml of hot saline (0.9% w/v of sodium chloride and freshly prepared distilled water) were added. The mixture was cooled , filtrated and the volume adjusted to 10 ml. with more saline solution. 5 ml of this solution was treated with few drops of ferric chloride solution. A dark blue colour was observed.

2.2.2.5 Test for Saponins

(1 g) of dried powdered plant material was placed in a clean test tube. 10 ml of distilled water were added and the tube was stoppered and vigorously shaken for about 30 seconds, The formation of a froth that persists for one hour indicates the presence of saponins.

2.2.3 Isolation of flavonoids from *Acacia Orefota*

(1Kg) Of powdered air-dried plant material was macerated with 80% aqueous methanol (5L) for 24hr. at room temperature with occasional shaking and then filtered off . The extraction process was repeated two more times with the same solvent. Combined filtrates were concentrated under reduced pressure using rotary evaporator at 40° C until all methanol was removed yielding a crude product, which was suspended in 300 ml water and left overnight in a refrigerator and then filtrated. The aqueous filtrate (500 ml.) was partitioned successively with n-hexane, chloroform, ethyl acetate and n-butanol.

Open column (80x 4 cm) was used for fractionation the nbutanol fraction. Polyamide was used as stationary phase and water /methanol (4:1 ; 3:2 ; 2:3 ;1:4 ;v:v:v) was used as mobile phase to afford collective fractions. Fraction 1 was further purified by paper chromatography to get two distinct purple zones which were purified via a sephadex column eluted with water: methanol (1:1.v:v) to yield compounds II and III.

2.2.4 Isolation of flavonoids from *Cordia sinensis*

(1Kg) Of powdered air-dried plant material was macerated with 80% aqueous methanol (5L) for 24hr. at room temperature with occasional shaking and then filtered off . The extraction process was repeated two more times with the same solvent. Combined filtrates were concentrated under reduced pressure using rotary evaporator at 40° C until all methanol was removed yielding a crude product, which was suspended in 300 ml water and left overnight in a refrigerator and then filtrated. The aqueous filtrate (500 ml.) was partitioned successively with dichloromethane and n-butanol .

Open dianion column (80x4cm) eluted with water: methanol (1:1) yielded six fractions.Fraction 5 was eluted successive with water : methanol (3:1 ; 1:1 ; 1:3 ; v:v) and methanol .

Sub-fraction (water:methanol; 3:1) was fractionated over a Sephadex LH20 column eluted successively with water: ethanol(4:1; 3:2;2:3;1:4 ;v:v) and ethanol to give three single spots of collective fractions on the basis of chromatographic properties on (PC). Colours of the spots ranged from blue to purple. The most intense purple colour was further purified through a Sephadex LH 20 column eluted with water: ethanol (1:1) to afford compound I.

For paper chromatography, spots were visualized under UV lights using both short and long wavelengths with and without exposure to NH_3 and sprayed with NA reagent.

2.2.5 Biological activity

2.2.5.1 Preparations of crude extracts for biological study

- Ethanol extract: prepared by macerating 100g of the air dried powdered plant material in successive portions of ethanol (100%) till exhaustion. The ethanolic extract was evaporated under reduced pressure to obtain a semi – solid residue.

- Ethyl acetate fraction was prepared by suspending the semisolid residue obtained from the ethanolic extract in the least amount of distilled water, then shaking with successive portions of ethyl acetate till exhaustion. The ethyl acetate extract was evaporated under reduced pressure to obtain the residue.

- n-Butanol fraction: prepared by suspending the semisolid residue obtained from the ethanolic extract in the least amount of distilled water, then shaking with successive portions of n-butanol till exhaustion. The n-butanol extract was evaporated under reduced pressure to obtain the residue.

-Chloroform fraction: prepared by suspending the semisolid residue obtained from the ethanolic extract in the least amount of distilled water, then shaking with successive portions of chloroform till exhaustion. The

chloroform extract was evaporated under reduced pressure to obtain the residue.

2.2.5.2 Antimicrobial assay

The methanolic extract and ethyl acetate fraction were screened for their antimicrobial activity against six standard human pathogens (*Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida albicans* and *Aspergillus niger*) using the cup plate agar method with some minor modifications.

2.2.5.2.1 Preparation of bacterial suspensions

One ml. aliquots of 24 hours broth culture of the test organisms were distributed onto agar slopes and incubated at 37°C for 24 hours.

The bacterial growth was harvested and washed off with sterile normal saline, and finally suspended in 100 ml of normal saline to produce suspension containing about 10^8 – 10^4 colony forming units per ml. The suspension was stored in refrigerator at 4°C until used. The average number of viable organism per ml of the saline suspension was determined by means of the surface viable counting technique.

Serial dilutions of the stock suspension were made in sterile normal saline in tubes and one drop volume (0.02 ml) of the appropriate dilutions were transferred by adjustable volume micropipette onto the surface of dried nutrient agar plates. The plates were allowed to stand for two hours at room temperature to dry, and then incubated at 37°C for 24 hours.

2.2.5.2.2 Preparation of fungal suspensions

Fungal cultures were maintained on dextrose agar incubated at 25°C for four days. The fungal growth was harvested and washed with sterile normal saline, and the suspension was stored in the refrigerator until used.

2.2.5.2.3 Testing for antibacterial activity

The cup plate agar diffusion method was adopted with some minor modification, to assess the antibacterial activity of the methanolic extract and ethyl acetate fraction of *Combretum aculeatum*. Two ml of the standardized bacterial stock suspension were mixed with 200 ml of sterile molten nutrient agar which was maintained at 45° C in water bath.

(20 ml) Aliquots of the incubated nutrient agar were distributed into sterile Petri dishes and the agar was left to settle in each of these plates which were divided into two halves. Two cups in each half (10 mm in diameter) were cut using sterile cork borer (No. 4). Each of the halves was designed for one of the extracts.

The agar discs were removed and cups were filled with (0.1) ml of each extract using adjustable volume microtiter pipette and allowed to diffuse at room temperature for two hours. The plates were then incubated in the upright position at 37 ° C for 24 hours.

The above procedure was repeated for different concentrations of the extracts and the standard antimicrobial chemotherapeutics. After incubation the diameters of the resultant growth inhibition zones were measures.

.2.5.2.4 Testing for antifungal activity

The above mentioned method was adopted for antifungal activity, but instead of nutrient agar dextrose agar was used. Samples were used here by the same concentrations used above.

3.Results and Discussions

3.1 Preliminary phytochemical screening

The bark of *Cordia senensis* was extracted with aqueous ethanol and the crude product was screened for secondary metabolites. Results are displayed in table 3. 1.

Table 3.1: Phytochemical screening of *Cordia senensis* bark extract

Phytochemicals	Reagent	ethanol extract
Alkaloids	Dragendroffs	+
Flavonoids	NaOH, FeCl ₃ , AlCl ₃ +	
Saponins	Foam test	-
Tannins	FeCl ₃ ; Salt gelatin test	+
Sterols/terpenes	Sakowski test Libermann test	+

The crude extracts of *Cordia senensis* and *Acacia oerofota* were fractionated by different chromatographic techniques. After the usual workup compound I was isolated from *Cordia senensis* , while compounds II and III were isolated from *Acacia oerfota*.

3.2 Spectral data of compound I

In UV, compound I absorbs (Fig.3.1) at λ_{\max} (MeOH) 268,331nm. Such absorption indicates conjugation between the 4 keto function and the B aromatic ring of the flavonoid nucleus. It is characteristic of flavones¹²⁶.

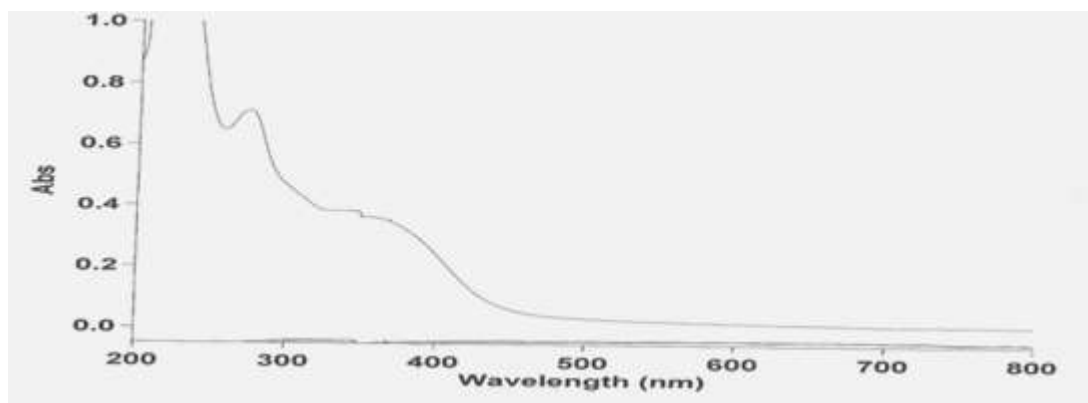
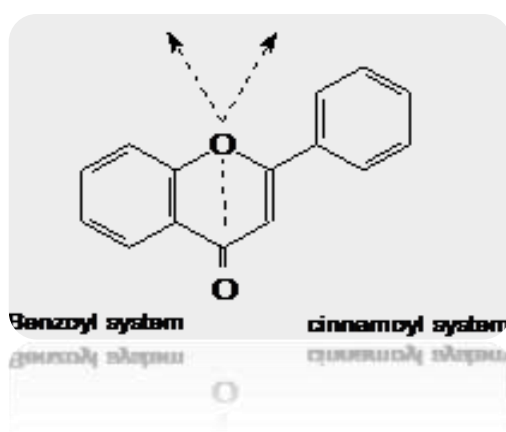
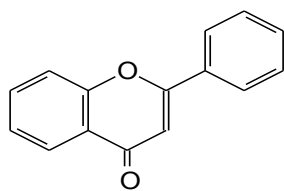


Fig.3.1 : UV spectrum of compound I

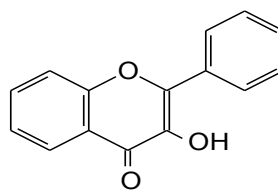
In their UV spectra flavones give both band I (due to cinnamoyl chromophore) and band II (due to benzoyl chromophore), a feature which is shared by flavonols, chalcones and aurones. Other classes: isoflavones, flavanones, dihydrochalcones and dihydroflavonols afford only one peak originating from the benzoyl system.

Band I, usually 300 – 400nm and band II, usually 240 – 280 nm¹²⁶.

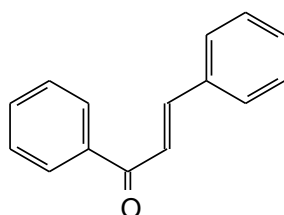




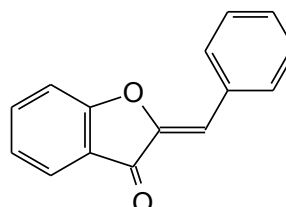
Flavone



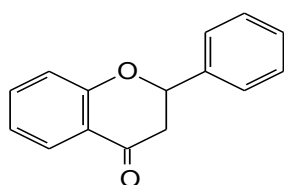
Flavonol



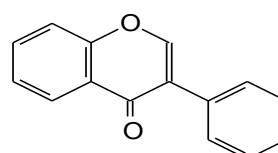
Chalcone



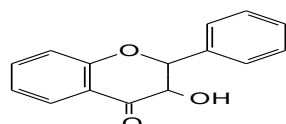
Aurone



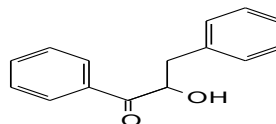
Flavanone



Isoflavone



Dihydroflavonol



Dihydrochalcone

The UV absorption of some important classes of flavonoids is depicted in table 3.2

Table (3.2): The UV. absorption of some flavonoids

Flavonoid class	Band I	Band II
Favone	330-350	250-270
Flavonol	350-390	250-280
Chalcone	365-390	240-260
Aurone	390-430	240-270

Very significant structural features have been obtained by utilizing the so-called UV shift reagents which produce shifts in the UV absorption maxima in accordance with the location of the various hydroxyl functions in flavonoid nucleus; these reagents are: sodium methoxide (which is diagnostic of 3- and 4'-OH functions); sodium acetate (diagnostic of 7-OH function); aluminium chloride (diagnostic of 3-, 5-OH and catechol systems) and boric acid (diagnostic of catechol systems)¹²⁶.

When sodium acetate was added to a methanolic solution of compound I, a bathochromic shift diagnostic of a 7-OH function was observed. Other shift reagents failed to give any detectable bathochromic shift.

The ¹HNMR spectrum (Fig.3.2) showed: δ 1.35ppm assigned for a methyl group; δ 3.36 characteristic of a methoxyl function; δ 3.50-4.64 assigned for sugar moiety; δ 6.22 assigned for C₆-proton. Other aromatic protons appeared at δ 6.47ppm, δ 6.94 and 7.40(B ring), while C₅ proton resonated as anticipated at lower field (δ 7.88) due to the deshielding influence of the neighboring 4 keto function. The EI mass spectrum (Fig.3.3) gave m/z 283 for M⁺ (aglycone).

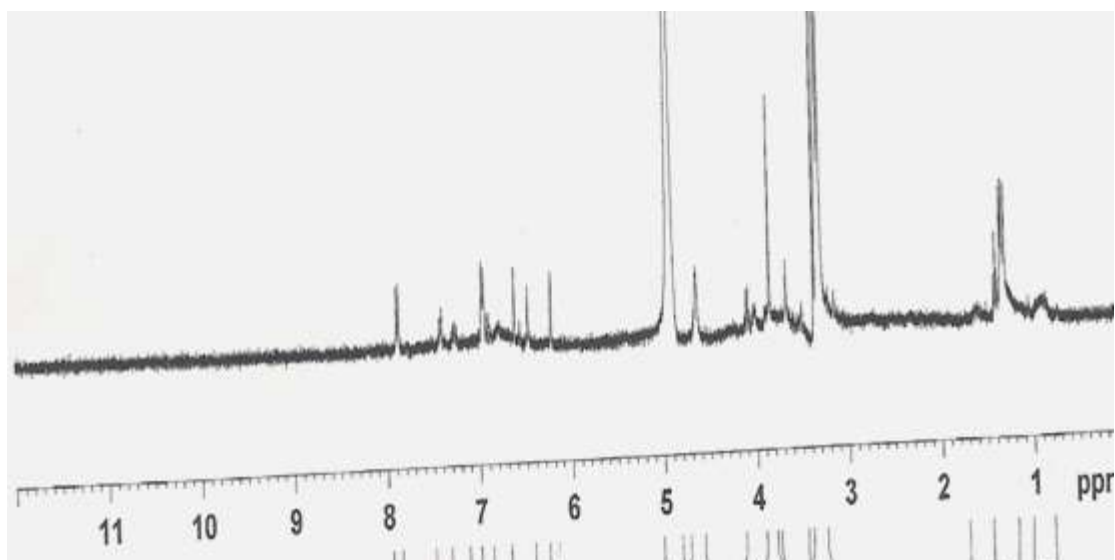


Fig.3.2 : ^1H NMR spectrum of compound I

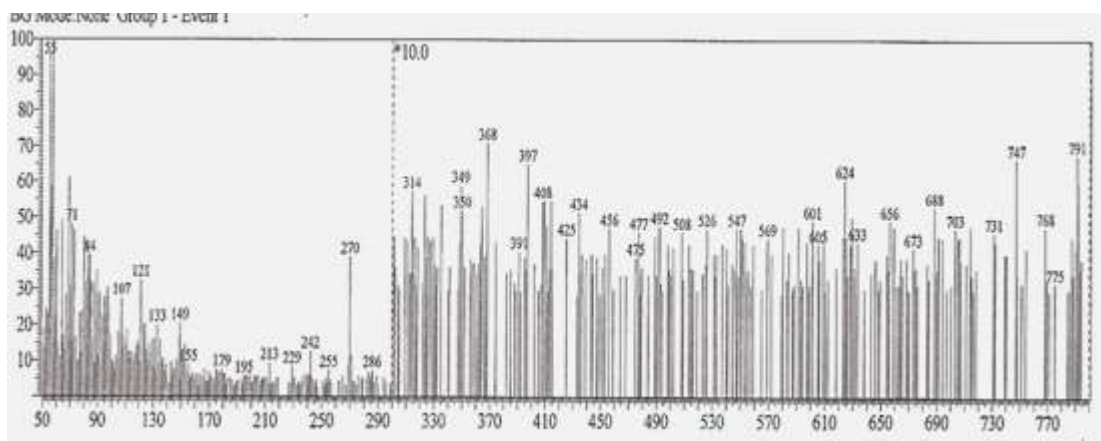
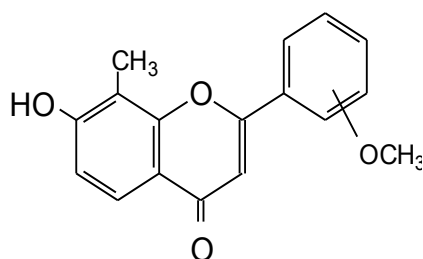
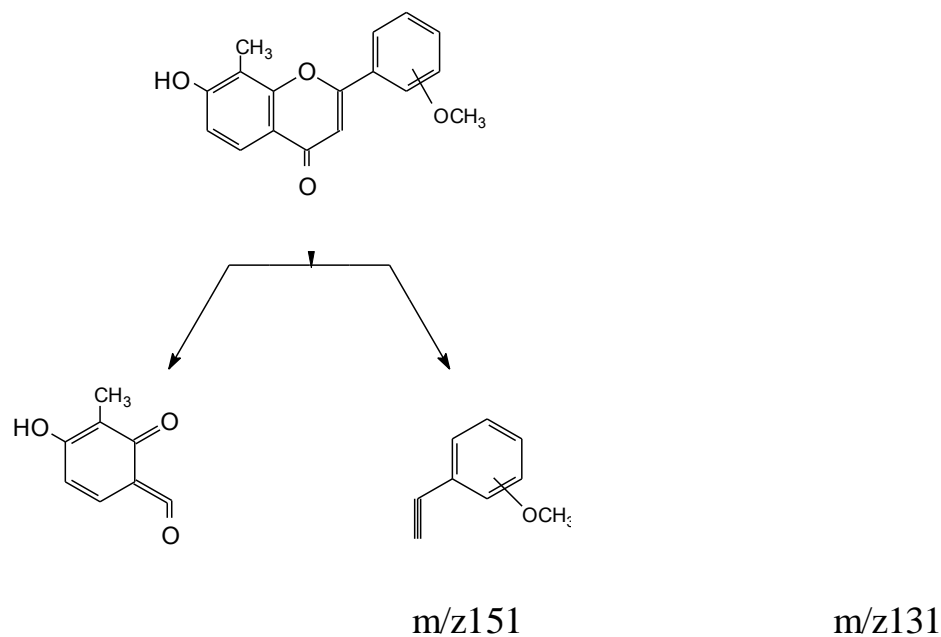


Fig.3.3 : Mass spectrum of compound I

Two important fragments resulting from retro Diels – Alder fission and corresponding to intact A and B rings appeared at m/z 151 and m/z 131. This lends evidence for the following partial structure for aglycone of compound I:



Compound I



Scheme I : Retro Diels-Alder fission of compound I

3.3 Characterization of compound II

Compound II was isolated as yellow amorphous powder from *Acacia oerfota*. The structure of this flavonoid was deduced on the basis of its spectral data (IR,UV,HNMR and MS).

In UV , compound II absorbs at λ_{max} (MeOH) 261,272,360nm(Fig.3.4). Such absorption is characteristic of flavonols¹²⁶.

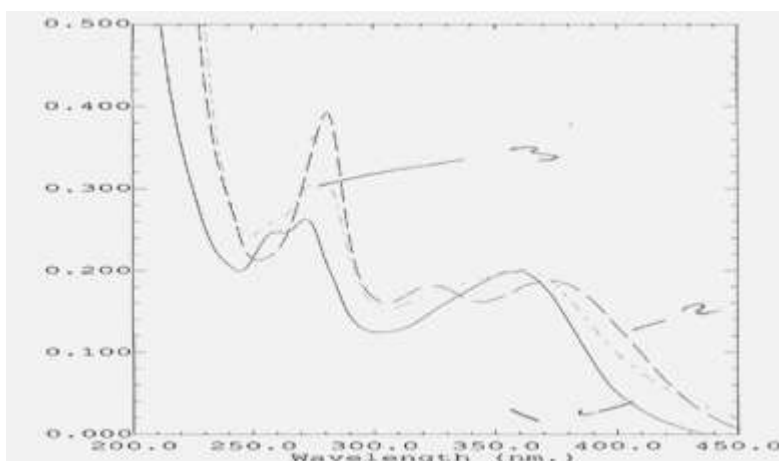


Fig.3.4 : UV spectrum of compound II

The sodium acetate spectrum (Fig.3.4) revealed a bathochromic shift diagnostic of a 7-OH function. Other shift reagents did not afford any detectable bathochromic shift.

The ^1H NMR spectrum (Fig.3.5) showed: δ 1.33(3H) assigned for a methyl group; δ 1.2.5(3H) accounting for an acetyl function; δ 3.35-3.86 attributed to a sugar moiety; δ 3.96(9H) assigned for three methoxyl groups; δ 6.21(1H) accounting for C₆-proton; δ 7.91(1H) attributed to C₅-proton. The resonance at δ 7.07 accounts for B ring protons.

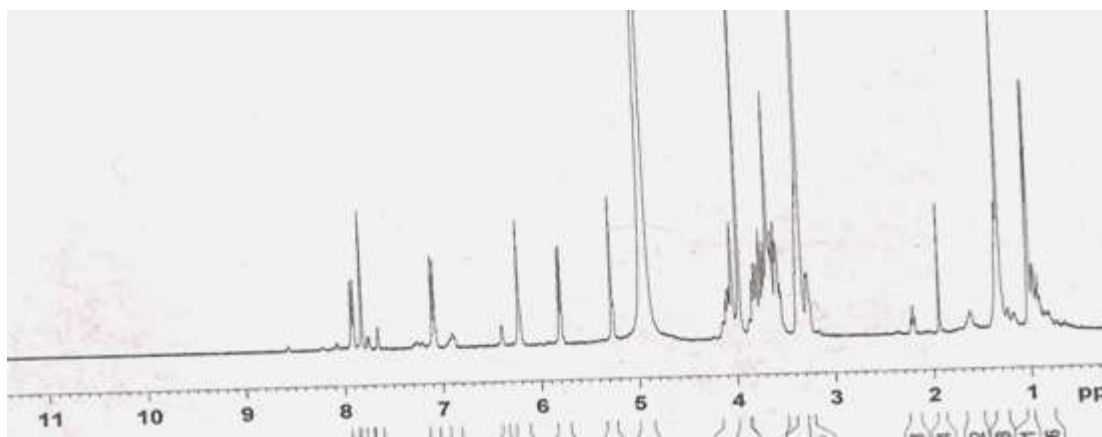


Fig. 3.5 : ^1H NMR spectrum of compound II

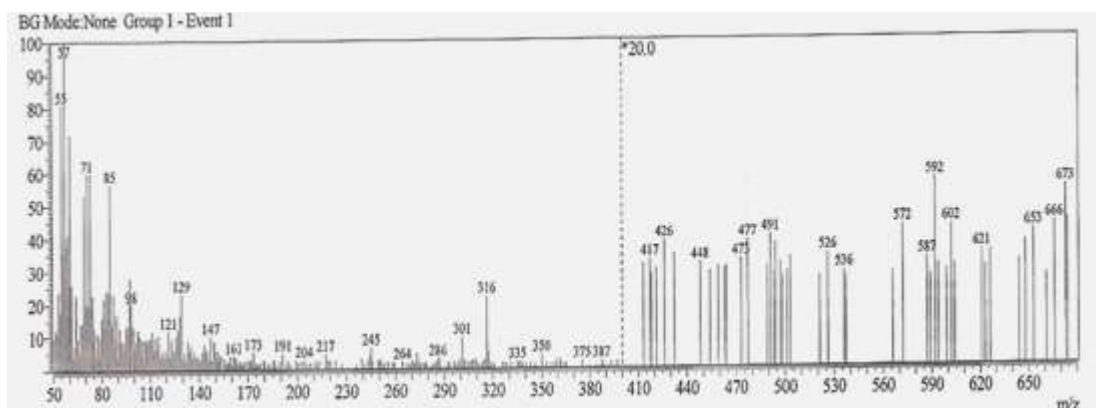
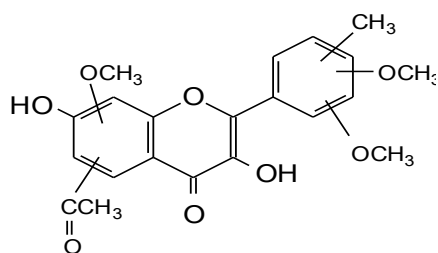
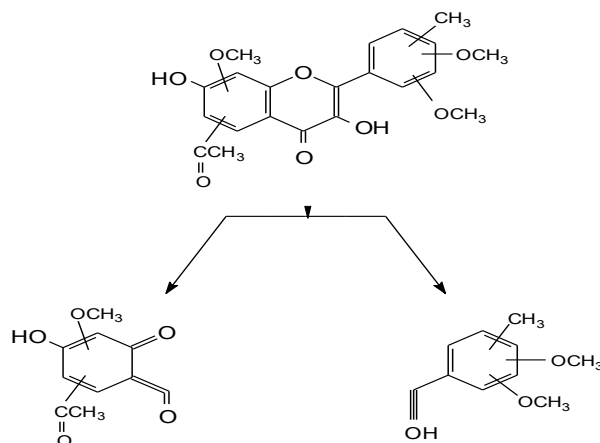


Fig. 3.6 : Mass spectrum of compound II

The mass spectrum (Fig.3.6) gave m/z 400 for M^+ (aglycone). The retro Diels-Alder cleavage gave m/z 185 for intact A ring. On the basis of the above cumulative data the following partial structure was proposed for aglycone of compound II:



II



m/z208

m/z196

Scheme II : Retro Diels-Alder fission of compound II

3.4 Characterization of compound III

Compound III was isolated as pale yellow powder from *Acacia oerfota*. The structure of this isolate was elucidated on the basis of its spectral data (IR, UV, ^1H NMR and MS).

The UV spectrum of compound III gave λ_{max} (MeOH) 272,323, 354nm (Fig.3.7). Such absorption is usually given by flavones¹²⁶. The sodium acetate spectrum (Fig.3.8) revealed a bathochromic shift diagnostic 7-OH function. No detectable bathochromic shifts were observed with other shift reagents.

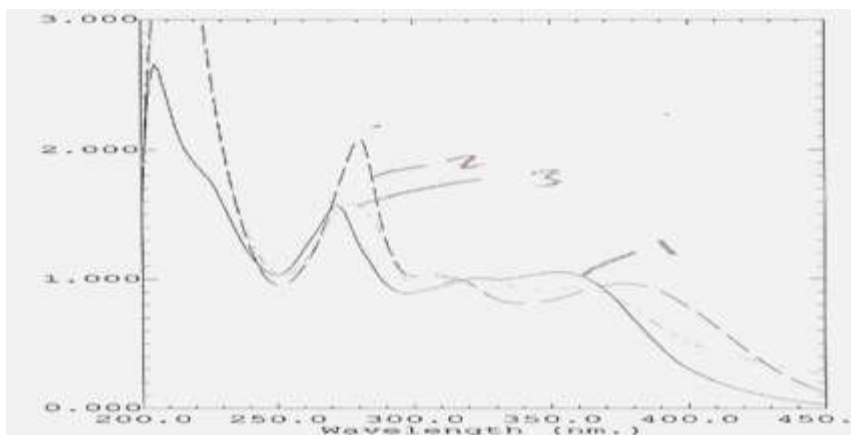


Fig.3.8 : UV spectrum of compound III

The ^1H NMR spectrum(Fig.3.9) showed: δ 01.00 integrating for 6 protons assigned for two methyl groups; δ 3.63(3H) and δ 3.80(3H) accountin for two methoxyl functions ; δ 5.96, δ 7.04 assigned for C_6 - and C_8 -protons respectively. The B ring aromatic protons appeared at δ 6.94 and δ 8.19ppm . The mass spectrum(Fig.) gave m/z 326 for M^+ (aglycone).

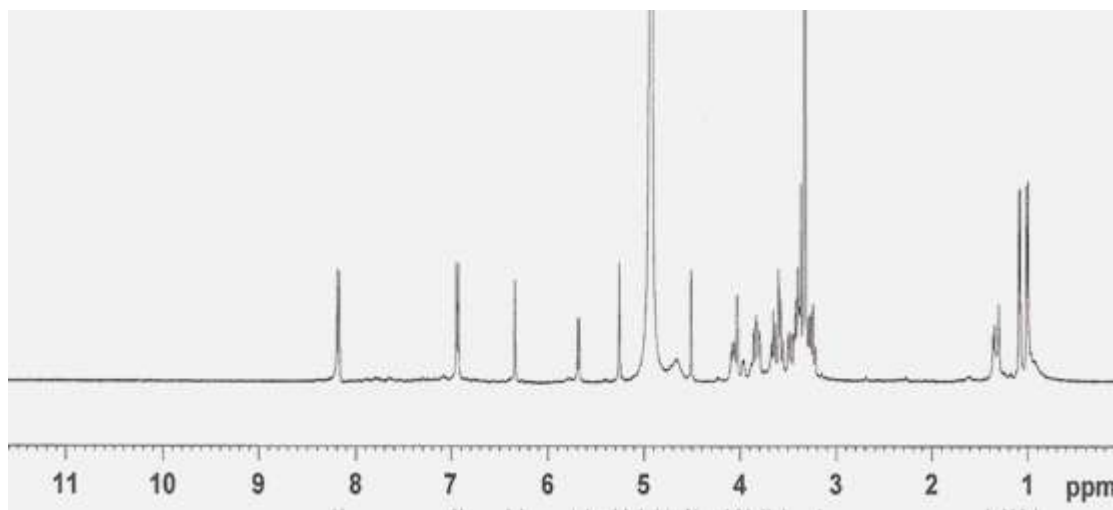


Fig. 3.9 : ^1H NMR spectrum of compound III

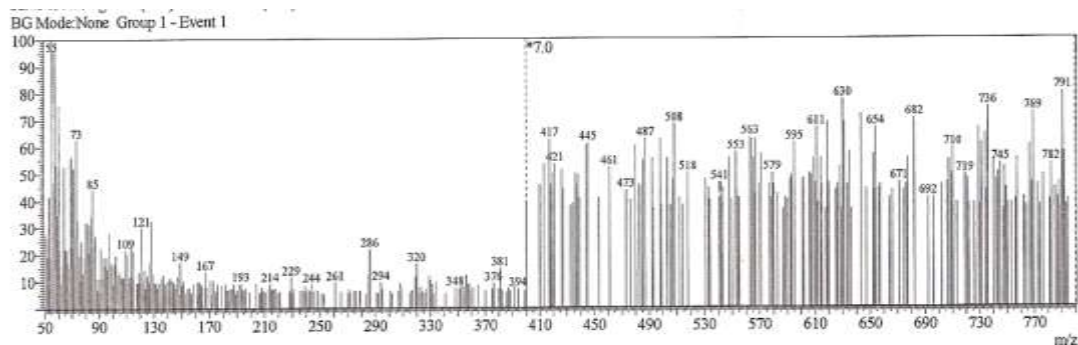
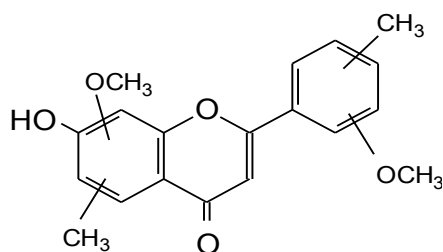
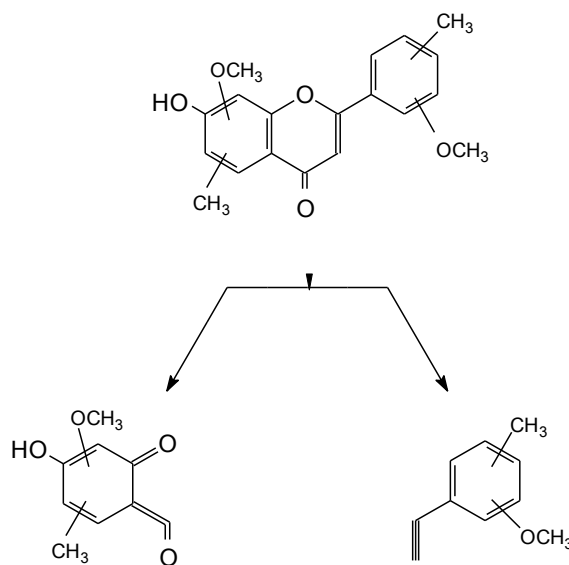


Fig. : Mass spectrum of compound III

The assignment of substituents for A and B rings was based on the retro Diels-Alder cleavage (Scheme III) which revealed signals at m/z 179 and m/z 144 for intact A and B rings respectively. Comparison of the above cumulative data with data published on literature resulted in the partial structure for compound III:



Compound III



m/z 163

m/z 145

Scheme III : Retro Diels-Alder fission of compound III

Bioassay

Results of antibacterial testing of the different concentrations of the extract are shown in Table (1). Different concentrations exhibited different antimicrobial responses against Gram positive and Gram negative bacteria, with zones of inhibition ranging from 6 to 13mm.

In addition, the extract showed good activity against *B. subtilis*, *P. aeruginosa* and *E. coli* with zones of inhibition of 13, 13 and 12 mm, respectively with minimum inhibitory concentrations (MIC) 62.5, 125 and 125 µg, respectively (Table 2). However it showed moderate inhibitory activity against the Gram positive bacterial pathogen *S. aureus* with zone of inhibition 9 mm.

Different concentrations of the extract were tested for their antifungal activity against *C. albicans* and five pathogenic fungi. The results are displayed in Table (2). The extract exhibited a moderate inhibitory activity against the yeast *C. albicans* in comparison with Treflucan.

Table (1): Antibacterial activity of different concentrations of the extract

Conc. (mg/5µL (DMSO/disc)	Gram positive bacteria		Gram negative bacteria	
	<i>B. subtilis</i> ATCC6633	<i>S. aureus</i> ATCC29213	<i>E. coli</i> ATCC 25922	<i>P.aeruginosa</i> ATCC27953
2.0	13	9	12	13
1.0	12	8	11	11

0.5	11	6	10	8
0.25	10	6	9	6
0.125	8	N.A.	7	6
Thiophenicol	22	11	15	14
Treflucan	N.A.	N.A.	N.A.	N.A.

Table (2): Antifungal activity of different concentrations of the extract

Fungi					
<i>C.albicans</i>	<i>A.niger</i>	<i>F.solani</i>	<i>F.oxysporium</i>	<i>A.alternata</i>	<i>A.tenuissima</i>
8	N.A.	N.A.	N.A.	N.A.	N.A.
7	N.A.	N.A.	N.A.	N.A.	N.A.
7	N.A.	N.A.	N.A.	N.A.	N.A.
6	N.A.	N.A.	N.A.	N.A.	N.A.
N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
8	13	11	15	20	22

Table (3): Minimal inhibitory concentration ($\mu\text{g}/\text{disc}$)

Entry	Gram positive bacteria		Gram negative bacteria		Fungi
	<i>B. subtilis</i> ATCC6633	<i>S. aureus</i> ATCC29213	<i>E. coli</i> ATCC25922	<i>P.aeruginosa</i> ATCC27953	<i>C.albicans</i> ATCC10321
Crude extract	62.5	250	125	125	250
Thiophenicol	3.13	3.13	25	25	-
Treflucan	-	-	-	-	25

Conclusion

The bark of *Cordia sinensis* was extracted with aqueous methanol and the crude product was screened for secondary metabolites. Results showed the presence of: flavonoids, tannins, alkaloids and sterols. Test for saponins was negative.

The crude extracts of *Cordia sinensis* and *Acacia Orefota* were fractionated by different chromatographic techniques. After the usual workup compound I was isolated from *Cordia sinensis*, while compounds II and III were isolated from *Acacia Orefota*.

In cup plate diffusion bioassay, different concentrations of total extract of *Acacia Orefota* exhibited different antimicrobial responses against Gram positive and Gram negative bacteria, with zones of inhibition ranging from 6 to 13 mm.

Recommendation

- i) A future ^{13}C NMR as well as 2D NMR experiments are recommended for full structural elucidation.
- ii) The isolated compounds may be screened for biological activities (antimicrobial, antimalarial, anti-inflammatory.....etc).
- iii) Other constituents (steroids, terpenoids, alkaloids.....etc) of the target species may be isolated and characterized.

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